D i s s e r t a t i o n

Phospholipid Biogenesis in the Apicomplexan Parasites *Eimeria falciformis* and *Toxoplasma gondii*

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Abstract

The survival and proliferation of apicomplexan parasites oblige efficient synthesis of phospholipids throughout their life cycles. Here, we first deployed *Eimeria falciformis* to investigate the process of lipid biogenesis in sporozoites. Lipidomics analyses demonstrate the occurrence of two exclusive lipids phosphatidylthreonine (PtdThr) and inositol phosphorylceramide along with other prototypical lipids. The parasite expresses nearly the entire lipid biogenesis network, which is an evolutionary mosaic of eukaryotic- and prokaryotic-type enzymes. Using *Toxoplasma gondii* as a gene-tractable surrogate to examine the *Eimeria* enzymes, we show a highly compartmentalized network of lipid synthesis distributed primarily in the apicoplast, ER, Golgi and mitochondrion. Likewise, trans-species complementation of a *T. gondii* mutant with a PtdThr synthase from *E. falciformis* suggests a convergent function of PtdThr in promoting the lytic cycle in coccidian parasites.

We also employed the well-established model parasite *T. gondii* to explore *de novo* synthesis and metabolic roles of one major lipid precursor CDP-diacylglycerol (CDP-DAG). We report the occurrence of two phylogenetically divergent CDP-DAG synthase (CDS) enzymes in *T. gondii.* Eukaryotic-type *Tg*CDS1 and prokaryotic-type *Tg*CDS2 reside in the ER and apicoplast, respectively. Conditional knockdown of *Tg*CDS1 severely attenuates parasite growth, which translates into a nearly complete loss of virulence in a mouse model. Residual growth of the *Tg*CDS1 mutant is abolished by subsequent deletion of *Tg*CDS2. Lipidomics analyses reveal significant and specific decline in phosphatidylinositol (PtdIns) and phosphatidylglycerol (PtdGro) upon loss of *Tg*CDS1 and *Tg*CDS2, respectively. Taken together, our work establishes a phospholipid biogenesis model involving significant inter-organelle cooperation and lipid trafficking in apicomplexan parasites.

Keywords: apicomplexan parasite, *Toxoplasma*, *Eimeria*, phospholipid biogenesis

Zusammenfassung

Das Überleben und die Vermehrung der parasitär lebenden Apicomplexa setzen eine effiziente Synthese von Phospholipiden während ihres gesamten Lebenszyklus voraus. In dieser Arbeit nutzten wir zunächst *Eimeria falciformis* um den Prozess der Lipid-Biogenese in Sporozoiten zu untersuchen. Durch Lipidomics-Analysen wurde das Auftreten von zwei exklusiven Lipiden, Phosphatidylthreonin (PtdThr) und Inositolphosphorylceramid. Der Parasit exprimiert fast das gesamte Lipid-Biogenese-Netzwerk aus eukaryotischen und prokaryotischen Enzymen. *Toxoplasma gondii* diente als genmanipulierbarer Ersatz für die Untersuchung der *Eimeria*-Enzyme, mit dem wir ein stark räumlich segmentiertes Netzwerk der Lipidsynthese im Apicoplast, ER, Golgi und Mitochondrium zeigen konnten. Ebenso legte die Komplementierung einer *T. gondii*-Mutante mit einer PtdThr-Synthase von *E. falciformis* eine konvergente Funktion von PtdThr für den lytischen Zyklus von Kokzidien-Parasiten nahe.

Außerdem setzten wir *T. gondii* als etablierten Modelorganismus ein, um die *De-novo*-Synthese und die metabolische Rolle eines bedeutenden Lipidvorläufers, CDP-Diacylglycerin (CDP-DAG), zu untersuchen. Wir konnten zwei phylogenetisch divergente CDP-DAG-Synthase (CDS) Enzyme in *T. gondii* nachweisen. Das eukaryotisch-typische *Tg*CDS1 und das prokaryotisch-typische *Tg*CDS2 lokalisieren im ER bzw. im Apicoplast. Der konditionierte Knockdown von *Tg*CDS1 bremst das Parasitenwachstum stark ab, was den fast vollständigen Verlust der Virulenz im Mausmodell hervorruft. Das restliche marginale Wachstum der *Tg*CDS1 Mutante wird durch zusätzliche Deletion der *Tg*CDS2 verhindert. Lipidomics-Analysen zeigten eine signifikante und spezifische Abnahme der Phosphatidylinositol (PtdIns)- und Phosphatidylglycerol (PtdGro)-Level bei Verlust der *Tg*CDS1- bzw. *Tg*CDS2-Gene. Zusammengenommen zeigt unsere Arbeit ein Phospholipid-Biogenese-Modell mit erstaunlicher Kooperation verschiedener Organellen und einem extensiven Lipidtransport im Parasiten.

Schlüsselwörter: Apikomplexan-Parasit, *Toxoplasma*, *Eimeria*, Phospholipid-Biogenese

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List of abbreviations

Lipids and their precursors

CDP-DAG cytidine diphosphate-diacylglycerol
DAG diacylglycerol
EPC ethanolamine phosphorylceramide
Glycerol-3P glycerol 3-phosphate
GPI glycosylphosphatidylinositol
IPC inositol phosphorylceramide
Lyso-PtdOH lysophosphatidic acid
MUFA monounsaturated fatty acid
PIP phosphatidylinositol phosphate
PtdCho phosphatidylcholine
PtdEtn phosphatidylethanolamine
PtdGro phosphatidylglycerol;
PtdGro-P phosphatidylglycerol phosphate
PtdIns phosphatidylinositol
PtdOH phosphatidic acid
PtdSer phosphatidylserine
PtdThr phosphatidylthreonine
PUFA polyunsaturated fatty acid
SM sphingomyelin

Enzymes of lipid synthesis

CDS cytidine diphosphate-diacylglycerol synthase
CEPT choline/ethanolamine phosphotransferase
CLS cardiolipin synthase
DGK diacylglycerol kinase
EPT ethanolamine phosphotransferase
FAE fatty acid elongase
FASII type II fatty acid synthase
G3PAT glycerol 3-phosphate acyltransferase
LPAAT lysophosphatidic acid acyltransferase
PAP phosphatidic acid phosphatase
PEMT phosphatidylethanolamine N-methyltransferase
PGPP phosphatidylglycerol phosphate phosphatase
PGPS phosphatidylglycerol phosphate synthase
PIS phosphatidylinositol synthase
PSD phosphatidylserine decarboxylase
PSS phosphatidylserine synthase
PTS phosphatidylthreonine synthase

Subcellular locations and markers

DER1 degradation in the endoplasmic reticulum protein 1
DG dense granule
ER endoplasmic reticulum
ERD2 endoplasmic reticulum deficient mutant 2
F1B ATPase subunit F1-B
Fd ferredoxin
GAP45 glideosome-associated protein 45
GRA1 dense granule protein 1
PV parasitophorous vacuole
SAG1 surface antigen 1

Selection markers and drugs

CAT chloramphenicol acetyltransferase
DHFR-TS dihydrofolate reductase-thymidylate synthase
FUDR 5-Fluoro-2'-deoxyuridine
HXGPRT hypoxanthine-xanthine-guanine phosphoribosyltransferase
MPA mycophenolic acid
UPRT uracil phosphoribosyltransferase

Chemicals and media

ATc anhydrotetracycline
BSA bovine serum albumin fraction V
DMEM Dulbecco's modified eagle media
DMSO dimethyl sulfoxide
DTT 1,4-Dithiothreitol
FBS fetal bovine serum
HBSS Hank's balanced salt solution
PBS phosphat buffered saline

Real-time PCR references

EFA elongation factor A
GT1 glucose transporter 1
TubA tubulin A

Others

HFF human foreskin fibroblast
HPLC high-performance liquid chromatography
MS mass spectrometry

1. Introduction

1.1. Apicomplexan parasites

The protozoan phylum Apicomplexa comprises >6000 extant species of obligate intracellular parasites, most of which are well known for the possession of an apical complex structure required for host cell invasion [1-4], as well as a unique plastid-like organelle apicoplast acquired by secondary endosymbiosis of red algae [5-8]. These parasites infect a wide range of organisms including livestock and humans. Some of the most prevalent and representative apicomplexan parasites infecting mammals include *Plasmodium*, *Toxoplasma*, *Eimeria* and *Cryptosporidium*. These parasites together impose a significant healthcare burden and socioeconomic impact globally.

Most apicomplexans have complex lifecycles occurring in either one (*e.g. Eimeria* and *Cryptosporidium*) or more (*e.g. Plasmodium* and *Toxoplasma*) host organisms. The natural lifecycle comprises asexual and sexual reproduction gyrating often between the primary (*i.e.* definitive or sexual) and secondary (*i.e.* intermediate or asexual) hosts. Infective stages of the apicomplexans are termed zoites, which are formed after sexual (sporozoite) or asexual (merozoite, tachyzoite, *etc.*) developments (Figure 1). Infection of a host typically starts with ingestion of the sporulated oocysts following the sporogony process, which leads to sporozoite release and invasion of host cells. After proliferation, parasites break out of the host cell and infect neighboring cells as merozoites. This schizogony/merogony proliferation usually occurs several times until gametogony takes place, which eventually leads to the formation of new oocysts. Beside sporozoites and merozoites, another infectious form of parasites has been described as tachyzoites during the asexual development of *Toxoplasma gondii* [9] (Figure 1B).

1.1.1. *Eimeria*

The genus *Eimeria* consists of >1800 species of diverse vertebrate hosts and represents the largest clade in the phylum Apicomplexa [10]. *Eimeria* species are best known for their monoxenous (single host) lifecycle and high host specificity (Figure 1A). Upon ingestion of sporulated oocysts present in the environment, sporozoites are released and infect host intestinal epithelial cells. Then parasites develop into

merozoites and undergo several rounds of proliferation by schizogony. Eventually, merozoites differentiate into male and female gametes, which fertilize to yield oocysts. Oocysts are shed into environment, where each oocyst is sporulated to produce 8 sporozoites for subsequent transmission to the next host. The egress of merozoites and oocysts causes the lytic collapse of intestinal epithelia, leading to severe coccidiosis, which is potentially fatal to young animals. This causes substantial economic losses in husbandry worldwide [11,12]. *Eimeria tenella* is the most studied species because it infects the poultry [13-17]. Some other species of *Eimeria* parasitizing mouse (including *E. falciformis*) are particularly attractive to examine *in vivo* parasite-host interactions and mucosal infection immunology due to well-established utility of the host as a model organism [18-29]. However, *in vitro* culture of *Eimeria* species and genetic manipulation have not been established yet.

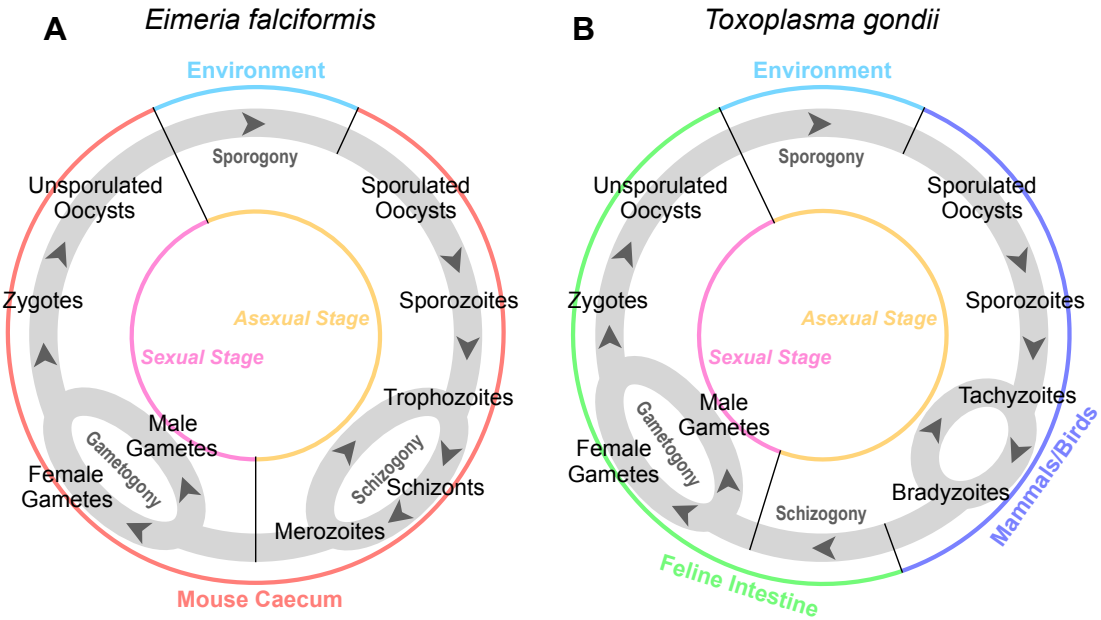


Figure 1. Life cycles of coccidian parasites *Eimeria falciformis* and *Toxoplasma gondii*. (**A**) *E. falciformis* completes its entire monoxenous life cycle in the mouse. After ingestion by the host, 8 sporozoites are released from each sporulated oocyst and invade the epithelial cells of the mouse caecum. Asexual schizogony takes place when the parasites transform into trophozoites and schizonts, which leads to merozoite production. Merozoites then burst out of the host cell and invade neighboring cells to start the next round of schizogony. Following 3-4 rounds of schizogony, parasites start sexual development into male and female gametes through gametogony process, leading to the formation of zygote and eventually unsporulated oocysts. The oocysts are shed into the environment where sporulation takes place. Mature oocysts are formed after sporogony and start the next life cycle in a new host. (**B**) The heteroxenous life cycle of *T. gondii* occurs between intermediate hosts (mostly mammals and birds) and definitive hosts (felids). The asexual development of parasites begins when sporozoites differentiate into rapid-proliferating tachyzoites and lead to a short acute infection in the intermediate hosts. Tachyzoites then transform into slow-growing bradyzoites, which persist as cysts mainly in brain, liver and muscle tissue. Bradyzoites could differentiate back to tachyzoites when host immunity is suppressed. After cats ingest the infected tissues of intermediate hosts, parasites undergo schizogony process, followed by sexual proliferation to complete the life cycle.

1.1.2. *Toxoplasma*

The genus *Toxoplasma* contains only one species *T. gondii*, and yet remains among the most cosmopolitan parasites in the phylum Apicomplexa. *T. gondii* is able to infect and reproduce in most nucleated cells of virtually all warm-blood organisms. Infection, pathogenesis and transmission of *T. gondii* depend on reversible switching of two asexual stages, tachyzoites and bradyzoites, causing acute and chronic infection, respectively (Figure 1B) [9]. Although usually asymptomatic in healthy human adults, the latent infection has recently been reported to be associated with altered behavioral changes and neurological disorders [30,31]. The parasite infection of developing fetus and individuals with deteriorated immunity can be potentially fatal due to severe tissue necrosis (acute infection), which is caused by successive rounds of lytic cycles of the tachyzoites that duplicate rapidly through endodyogeny process (Figure 2). Thanks to the well-annotated genome [32,33] and advanced methods for genetic manipulation [34-40], *T. gondii* has become an excellent model to study the biology of parasites and free-living protozoans.

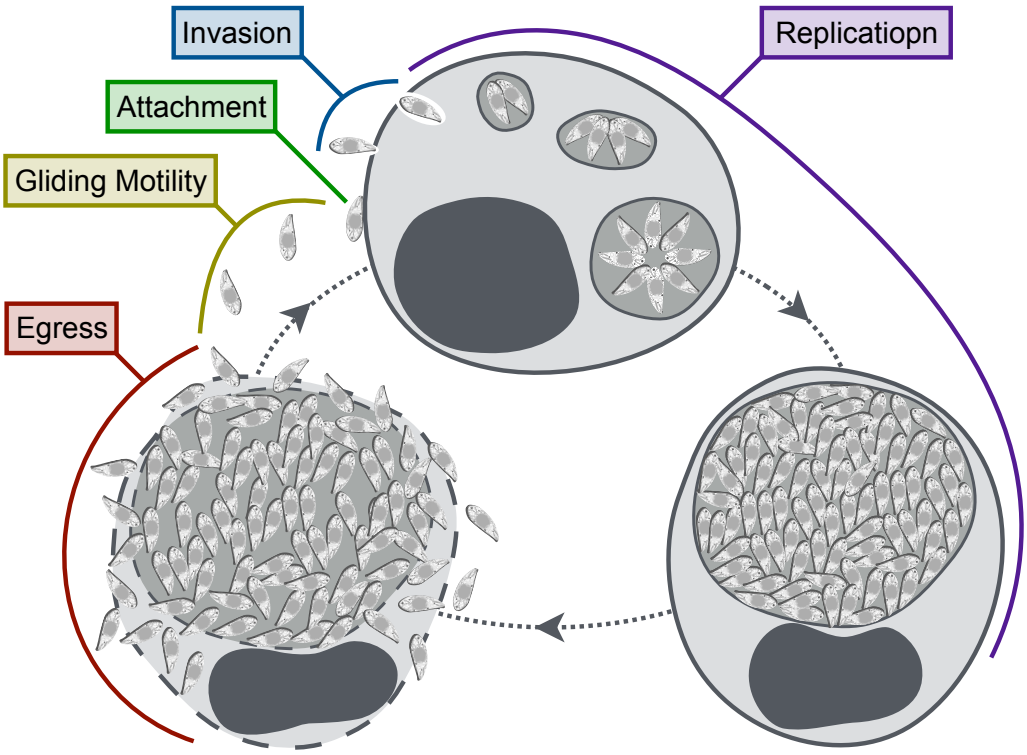


Figure 2. Lytic cycle of *T. gondii* tachyzoites. The lytic cycle starts when tachyzoites attach and invade a host cell, followed by rapid replication in a parasitophorous vacuole (PV) to generate new tachyzoites. PV and host cell are eventually lysed when tachyzoites egress, glide to neighbor cells and initiate the next cycle.

1.2. Lipid biology of apicomplexan parasites

Lipids are essential components of all cell types in every organism, and are particularly important for intracellular pathogens to survive and proliferate in their host cells. In apicomplexan parasites, phospholipids are the main structural components of both plasma membrane and intracellular membranes of compartmentalized organelles [41-47]. Phospholipids and their downstream products also serve as crucial pathogenesis factors by facilitating lytic cycles and escaping host immune responses [48-50]. Thus, parasites impose a substantial demand for phospholipids throughout their life cycles. Although apicomplexans have been shown to salvage lipids from host cells [51-54], recent genetic and biochemical studies have proven that *de novo* phospholipid synthesis is often essential for parasite viability and virulence at different life stages [50,55-59].

1.2.1. Phospholipid biogenesis in prokaryotes and eukaryotes

De novo synthesis of main phospholipids commences with the assembly of lysophosphatidic acid (Lyso-PtdOH) and phosphatidic acid (PtdOH) using glycerol 3-phosphate (Glycerol-3P) and fatty acids (Figure 3). In prokaryotes, PtdOH is converted into CDP-diacylglycerol (CDP-DAG) that serves as a substrate to synthesize all lipids (Figure 3). In eukaryotes, PtdOH functions as a precursor for both CDP-DAG and diacylglycerol (DAG) that subsequently enable the synthesis of distinct phospholipid classes. CDP-DAG is utilized to make phosphatidylinositol (PtdIns) and phosphatidylglycerol (PtdGro), whereas DAG drives the synthesis of phosphatidylcholine (PtdCho) and phosphatidylethanolamine (PtdEtn) *via* the CDP-choline and CDP-ethanolamine pathway, respectively. PtdEtn can also be generated by decarboxylation of phosphatidylserine (PtdSer), which itself is either derived by a base-exchange reaction from PtdEtn or PtdCho (mammals) or produced by fusion of CDP-DAG and serine (yeast). In some eukaryotic cells, such as yeast and mammalian hepatocytes, PtdEtn can also be methylated to yield PtdCho (Figure 3).

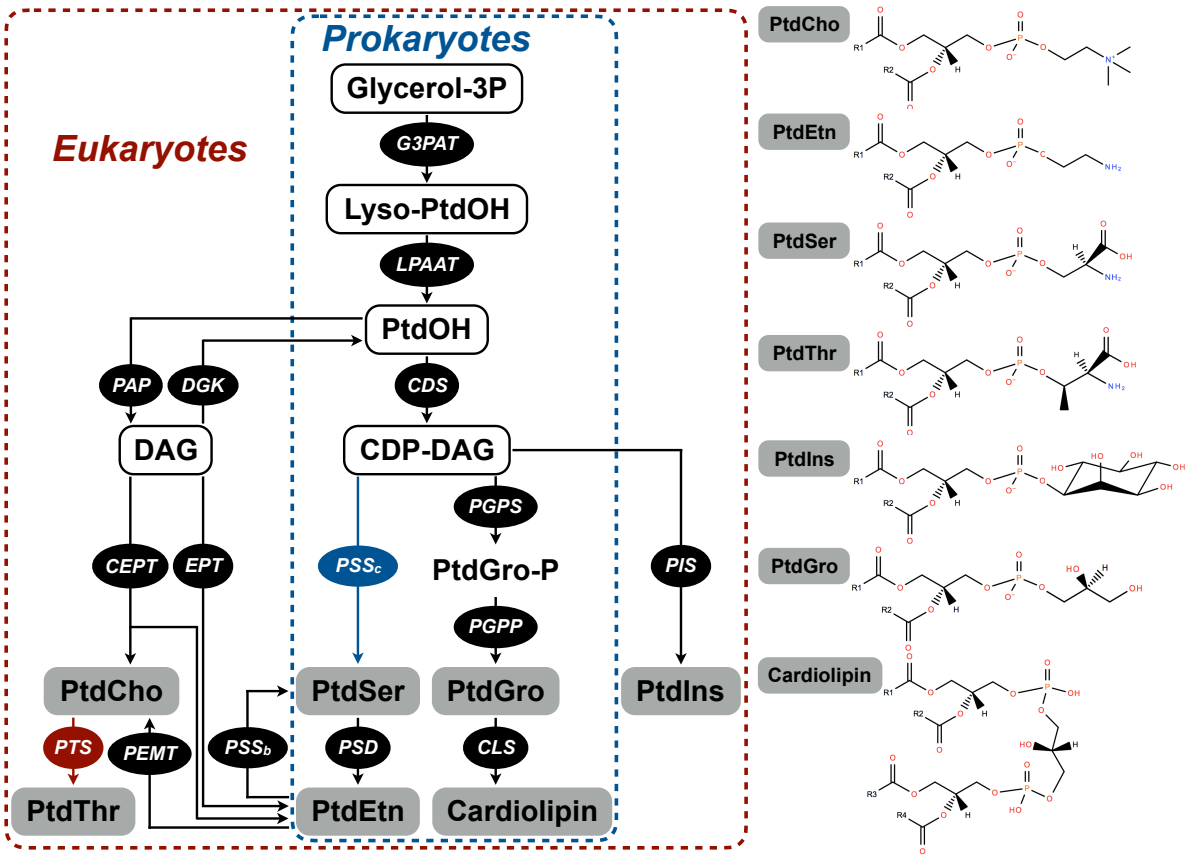


Figure 3. Synthesis of major phospholipids in prokaryotes and eukaryotes. The depicted pathways with black arrows in red box are present in mammalian cells (human/mouse), whereas bacteria harbor only the part of the network in blue box (including PSS_c shown with blue arrow). PTS is recently identified in apicomplexan parasite *T. gondii*, and is shown in red arrow. Major phospholipids, intermediates of their synthesis and underlying enzymes are in grey, white and black background, respectively. The structures of major phospholipids are shown on the right side to the network. *Abbreviations*: CDP-DAG, cytidine diphosphate-diacylglycerol; CDS, cytidine diphosphate-diacylglycerol synthase; CEPT, choline/ethanolamine phosphotransferase; CLS, cardiolipin synthase; DAG, diacylglycerol; DGK, diacylglycerol kinase; EPT, ethanolamine phosphotransferase; G3PAT, glycerol 3-phosphate acyltransferase; Glycerol-3P, glycerol 3-phosphate; LPAAT, lysophosphatidic acid acyltransferase; Lyso-PtdOH, lysophosphatidic acid; PAP, phosphatidic acid phosphatase; PEMT, phosphatidylethanolamine N-methyltransferase; PGPP, phosphatidylglycerol phosphate phosphatase; PGPS, phosphatidylglycerol phosphate synthase; PIS, phosphatidylinositol synthase; PSD, phosphatidylserine decarboxylase; PSS_b, phosphatidylserine synthase (base-exchange type); PSS_c, phosphatidylserine synthase (CDP-DAG-dependent type); PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; PtdGro, phosphatidylglycerol; PtdGro-P, phosphatidylglycerol phosphate; PtdIns, phosphatidylinositol; PtdOH, phosphatidic acid; PtdSer, phosphatidylserine; PtdThr, phosphatidylthreonine; PTS, phosphatidylthreonine synthase.

1.2.2. Phospholipid biogenesis in *T. gondii*

The lipid biogenesis in *T. gondii* initiates with the production of short acyl chains through a prokaryotic-like type II fatty acid synthase (FASII) pathway located in the apicoplast [60,61]. These acyl chains could be exported to the endoplasmic reticulum (ER), where they are modified through the fatty acid elongase (FAE) pathway to generate longer-chained and unsaturated fatty acids [62]. Glycerol-3P and fatty acids are utilized to synthesize Lyso-PtdOH and PtdOH, presumably in the apicoplast and/or ER [63], which subsequently facilitate the synthesis of major phospholipids.

The *T. gondii* membranes consist of polar lipids and neutral lipids [45,55]. We have shown that phospholipids account for a major fraction of total membrane lipids isolated from *T. gondii* tachyzoites. PtdCho is the most abundant phospholipid present in tachyzoites, followed by PtdEtn, phosphatidylthreonine (PtdThr), PtdIns, PtdSer, PtdGro and PtdOH [50,55]. Additionally, our previous work identified the endogenous routes of PtdCho, PtdEtn, PtdThr and PtdSer synthesis in the parasite (Figure 4) [50,64-66]. Synthesis of PtdCho, PtdThr and PtdSer occurs in the ER, whereas PtdEtn can be made in ER, mitochondrion, dense granule (DG) and parasitophorous vacuole (PV).

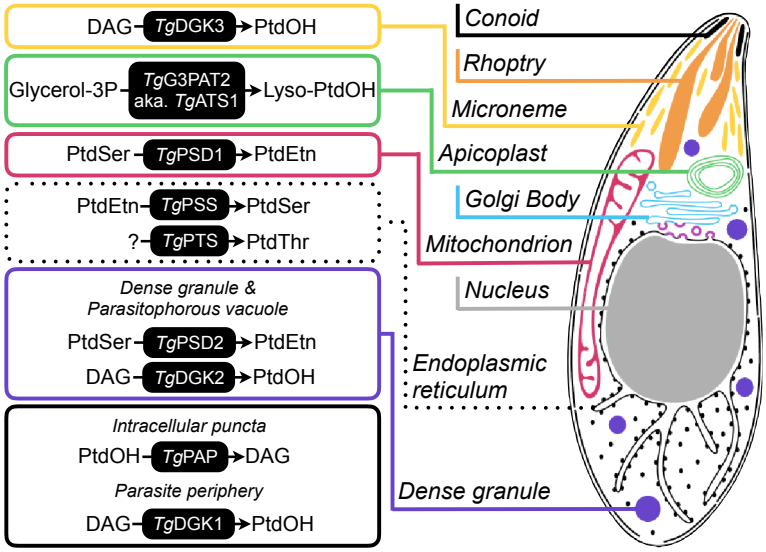


Figure 4. Summary of the lipid biogenesis enzymes identified in *T. gondii*. Several enzymes responsible for the synthesis of major lipids (PtdEtn, PtdSer and PtdThr) [50, 65-66] and their precursors (Lyso-PtdOH, PtdOH and DAG) [63, 118] are identified in various subcellular compartments in *T. gondii* tachyzoites as indicated in the figure. The sketch of *T. gondii* subcellular structure is modified from *reference 126*. *Abbreviations*: DAG, diacylglycerol; DGK, diacylglycerol kinase; G3PAT/ATS, glycerol 3-phosphate acyltransferase; Glycerol-3P, glycerol 3-phosphate; Lyso-PtdOH, lysophosphatidic acid; PAP, phosphatidic acid phosphatase; PSD, phosphatidylserine decarboxylase; PSS, phosphatidylserine synthase; PtdEtn, phosphatidylethanolamine; PtdOH, phosphatidic acid; PtdSer, phosphatidylserine; PtdThr, phosphatidylthreonine; PTS, phosphatidylthreonine synthase.

1.3. Objective of this study

The formation of apicomplexan “zoites” during infectious stages obliges considerable lipid biogenesis when parasites replicate inside a host cell. Previous studies on *Toxoplasma* tachyzoite and *Plasmodium* merozoite have demonstrated that these parasites not only deploy host-derived precursors to synthesize the required phospholipids [50,55-59], but also competent in salvaging selected lipids from the sheltering host cells [51-53]. Unlike all other infectious stages of the natural lifecycle that parasitize corresponding host cells to ensure survival and reproduction, the sporozoite development occurs extracellularly and does not involve intimate interactions with host cells. Being outside the host milieu, it is not known however, how freely developing sporozoites satisfy their phospholipid demands. Moreover, from a conceptual viewpoint, the sporozoite stage imparts an excellent model to evaluate the “actual” metabolic potential of otherwise host-dependent organism. Despite the outlined importance, the sporozoite metabolism remains literally a black box from any representative apicomplexan parasite, principally because it is somewhat challenging to obtain sufficient amounts of pure sporozoites from non-model primary hosts. One aim of this study is to employ the common rodent parasite *E. falciformis* to discern the network design principles of phospholipid biogenesis in sporozoites.

In addition to an archetypal lipid network, apicomplexan pathogens have evolved many novel and often physiologically essential pathways. Some of them have originated by the endosymbiosis processes of their common ancestor with red alga, as confirmed by the existence of a chloroplast relict (termed as apicoplast) in most extant apicomplexan parasites [61,63,67-69]. Such divergent lipid synthesis pathways offer therapeutic targets to selectively inhibit the parasite reproduction. The enzyme CDP-DAG synthase (CDS) is one of the most central enzymes of lipid synthesis in both prokaryotes and eukaryotes. Given the functional integration of apicoplast with other organelles harboring lipid synthesis, understanding the mechanisms and importance of CDP-DAG synthesis is particularly interesting in apicomplexans. The other aim of this study is to explore the phylogenetic and metabolic roles of two divergent CDS enzymes identified in the well-established model organism *T. gondii*.

2. Materials and methods

2.1. Materials

2.1.1. Biological resources

E. falciformis oocysts	Bayer (Leverkusen, Germany)
T. gondii tachyzoites	
- RHΔ*ku80*-Δ*hxgprt* strain [40]	Vern Carruthers (University of Michigan)
- RHΔ*ku80*-TaTi strain [70]	Boris Striepen (University of Georgia)
- Δ*tgpts* starin [50]	Ruben D. Arroyo-Olarte (Humboldt University Berlin)
Human foreskin fibroblast (HFF) cells	Cell Lines Service (Eppelheim, Germany)
NMRI mouse	Charles River Laboratories (Wilmington, MA)
E. coli XL-1Blue strain	Stratagene (Heidelberg, Germany)

2.1.2. Vectors

pNTP3	Isabelle Coppens (John Hopkins University)
pG152	Markus Meissner (University of Glasgow)
pTKO-HXGPRT	John Boothroyd (Stanford University School of Medicine)
pTUB8-CAT	Dominique Soldati-Favre (University of Geneva)
pTUB8-TgDER1-GFP	Boris Striepen (University of Georgia)
pTETO7SAG1-UPKO	modified from *pNTP3*
pGRA1-UPKO	modified from *pTKO-HXGPRT*
pTKO-CAT	modified from *pTKO-HXGPRT*
pTKO-DHFR-TS	modified from *pTKO-HXGPRT*

2.1.3. Antibodies

α-*Tg*F1B (mouse) [71] 1:1000	Peter Bradley (University of California)
α-*Tg*Fd (rabbit) [72] 1:500	Frank Seeber (Robert-Koch Institute Berlin)
α-*Tg*GAP45 (rabbit) [73] 1:10000	Dominique Soldati-Favre (University of Geneva)
α-*Tg*GRA1 (mouse) [74] 1:500	Marie-France Cesbron-Delauw (CNRS- Grenoble)
α-*Tg*HSP90 (rabbit) [75] 1:10000	Sergio Angel (IIB-INTECH Argentina)
α-*Tg*SAG1 (mouse) [76] 1:1000	Jean-Francois Dubremetz (University of Montpellier)
α-HA (mouse) 1:3000	Sigma-Aldrich (St. Louis, MO)

α-HA (rabbit) 1:1000	Sigma-Aldrich (St. Louis, MO)
α-Myc (mouse) 1:5000	Sigma-Aldrich (St. Louis, MO)
α-Myc (rabbit) 1:1000	Sigma-Aldrich (St. Louis, MO)
α-Ty1 (Mouse) 1:50	Sigma-Aldrich (St. Louis, MO)
α-mouse IgG Alexa Fluor 488 (goat) 1:3000	Life Technologies (Waltham, MA)
α-mouse IgG Alexa Fluor 594 (goat) 1:3000	Life Technologies (Waltham, MA)
α-rabbit IgG Alexa Fluor 488 (goat) 1:3000	Life Technologies (Waltham, MA)
α-rabbit IgG Alexa Fluor 594 (goat) 1:3000	Life Technologies (Waltham, MA)
α-mouse IgG IRDye 800CW (goat) 1:20000	LI-COR Biosciences (Lincoln, NE)
α-rabbit IgG IRDye 680RD (goat) 1:20000	LI-COR Biosciences (Lincoln, NE)

2.1.4. Oligonucleotides

All oligonucleotides used in this study were supplied from Life Technologies (Waltham, MA) and are listed in Table 1.

Table 1. Oligonucleotides used in this study.

Primer name (Restriction site)	Primer sequence (Restriction site underlined)	Vector (Objective)
Ectopic expression of selected *E. falciformis* enzymes in *T. gondii* tachyzoites (Δ*ku80*-Δ*hxgprt* strain)		
*Ef*G3PAT1-F (*Nsi*I)	CTCATCATGCATTATTTCCTGGTTAAGTGGCTCTG	*pGRA1-UPKO* (Ectopic expression)
*Ef*G3PAT1-HA-R (*Pac*I)	CTCATCTTAATTAACTAAGCGTAATCTGGAACATCGTATGGGTAAATGACTTCCGTAGAACCGC	
*Ef*G3PAT2-F (*Sbf*I)	CTCATCCCTGCAGGGGGGCCCCTATTCGCTT	
*Ef*G3PAT2-HA-R (*Pac*I)	CTCATCTTAATTAAGCGTAATCTGGAACATCGTATGGGTAGCGCGAAGAGACAACGC	
*Ef*LPAAT1-F (*Nsi*I)	CTCATCATGCATGAGCCCTATAAAATTTTATGTTTTCTC	
*Ef*LPAAT1-HA-R (*Pac*I)	CTCATCTTAATTAAGCGTAATCTGGAACATCGTATGGGTAGAGGCCATGACCGCG	
*Ef*LPAAT2-F (*Nsi*I)	CTCATCATGCATCCCCTGCTGCCGCAG	
*Ef*LPAAT2-HA-R (*Pac*I)	CTCATCTTAATTAACTAAGCGTAATCTGGAACATCGTATGGGTACTGCAGTGCTGCCTGCTCT	
*Ef*CDS1-F (*Sbf*I)	CTCATCCCTGCAGGCGGAGGGGGTGAAAATGC	
*Ef*CDS1-HA-R (*Pac*I)	CTCATCTTAATTAAGCGTAATCTGGAACATCGTATGGGTAATCCCCAGCGGCCG	
*Ef*CDS2-F (*Nsi*I)	CTCATCATGCATCCCTGTGGCACCCCAC	
*Ef*CDS2-HA-R (*Pac*I)	CTCATCTTAATTAACTAAGCGTAATCTGGAACATCGTATGGGTACCGCCTCATTCCCTTTGTC	
*Ef*PAP-F	CTCATCATGCATAAGCCGACAGATCAAGATTTG	

(*Nsi*I) *Ef*PAP-HA-R (*Pac*I)	CTCATCTTAATTAACTAAGCGTAATCTGGAACATCGTATGGG TAAGCCCTGAGATCCACAACAA	
*Ef*DGK1-F (*Nsi*I) *Ef*DGK1-HA-R (*Pac*I)	CTCATCATGCATCTGTCCGTAAGGAGAGCAACT CTCATCTTAATTAACTAAGCGTAATCTGGAACATCGTATGGG TATGGATCAGCTTGCACAGC	
*Ef*DGK2-F (*Pst*I) *Ef*DGK2-HA-R (*Pac*I)	CTCATCCTGCAGGACTCCTGCTCCCGATATGG CTCATCTTAATTAACTAAGCGTAATCTGGAACATCGTATGGG TACTTTCGATCTCGGTATCTTAGCAT	
*Ef*PGPS-F (*Nsi*I) *Ef*PGPS-HA-R (*Pac*I)	CTCATCATGCATGCAGCGTCGAC CTCATCTTAATTAAGCGTAATCTGGAACATCGTATGGGTAGA GCAAAGATCCGAGGCC	
*Ef*CLS-F (*EcoR*V) *Ef*CLS-HA-R (*Pac*I)	CTCATCGATATCATGGTGCTGCTGCAGTCC CTCATCTTAATTAATCAAGCGTAATCTGGAACATCGTATGGG TAGCATCCGCAGAAGATGGG	
*Ef*PIS-F (*Nsi*I) *Ef*PIS-HA-R (*Pac*I)	CTCATCATGCATTCTATGGACAAGGGGTTGAGG CTCATCTTAATTAACTAAGCGTAATCTGGAACATCGTATGGG TAGTCCTCGGCCTTCCTCG	
*Ef*CEPT1-F (*Nsi*I) *Ef*CEPT1-HA-R (*Pac*I)	CTCATCATGCATATGGTGGCTGGGGCCT CTCATCTTAATTAAGCGTAATCTGGAACATCGTATGGGTAGT CGTCTTTGACCTGCGC	
*Ef*CEPT2-F (*Nsi*I) *Ef*CEPT2-HA-R (*Pac*I)	CTCATCATGCATGGGGGAGCCTTCGGG CTCATCTTAATTAAGCGTAATCTGGAACATCGTATGGGTATTC TGCCTTTCTGTCCTTTCG	
*Ef*PSD1-F (*Nsi*I) *Ef*PSD1-HA-R (*Pac*I)	CTCATCATGCATGGCTGTCGAATTCCGTTG CTCATCTTAATTAACTAAGCGTAATCTGGAACATCGTATGGG TAGTAATGCACGAATCTGCGTTC	
*Ef*PSD2-F (*Sbf*I) *Ef*PSD2-HA-R (*Pac*I)	CTCATCCCTGCAGGAGCTCTTTTGCTCCATTCTGTT CTCATCTTAATTAAGCGTAATCTGGAACATCGTATGGGTATT GCGGTTTTTCTAAGTCAAAG	
*Ef*PSS-F (*Nsi*I) *Ef*PSS-HA-R (*Pac*I)	CTCATCATGCATGACGTGCGGCGTCG CTCATCTTAATTAACTAAGCGTAATCTGGAACATCGTATGGG TAGCTGCTGCAACAATCTGAAG	
*Ef*PTS-F (*Nsi*I) *Ef*PTS-HA-R (*Pac*I)	CTCATCATGCATAGGGAACCCAAAGAACAAGC CTCATCTTAATTAAGCGTAATCTGGAACATCGTATGGGTAGG ATCCGTAGTAGTACTGCTTGG	
3' HA tagging of selected genes (Δ*ku80*-Δ*hxgprt* strain)		
*Tg*CDS1-COS-F *Tg*CDS1-COS-R	TACTTCCAATCCAATTTAATGCTCTGTCGAAGGGAAGC TCCTCCACTTCCAATTTTAGCCCGAAATTCGGTTTGTCC	*pG152* (Ligation independent cloning)
*Tg*CDS2-COS-F *Tg*CDS2-COS-R	TACTTCCAATCCAATTTAATGCTCTCCCGGTGTCTGTCGTCT TCCTCCACTTCCAATTTTAGCGGCCTGCATCTGCGGGAC	
*Tg*PGPS-COS-F	TACTTCCAATCCAATTTAATGCAGAAGGCGGAGAAGAG	

*Tg*PGPS-COS-R	TCCTCCACTTCCAATTTTAGCAAACAACCAGATTCCCTT	
Ectopic expression of *Tg*CDS1 and *Tg*CDS2 without N-terminal extensions and with C-terminal Myc tag (*Tg*CDS1 or *Tg*CDS2 3' tagging strains)		
*Tg*CDS1$^{398\text{-}1068}$-F (*Nsi*I) *Tg*CDS1-Myc-R (*Pac*I)	CTCATCATGCATCGCGTCCGGTCGCT CTCATCTTAATTAATCAGAGGTCTTCTTCGGAAATCAACTTCT GTTCCCGAAATTCGGTTTGTCCT	*pTKO-CAT* (Ectopic expression)
*Tg*CDS2$^{527\text{-}1044}$-F (*Sbf*I) *Tg*CDS2-Myc-R (*Pac*I)	CTCATCCCTGCAGGAGCATTTGCGAGTGAGCACTG CTCATCTTAATTAATCAGAGGTCTTCTTCGGAAATCAACTTCT GTTCGGCCTGCATCTGCGG	*pTKO-DHFR-TS* (Ectopic expression)
Ectopic expression of *Tg*CDS1 and *Tg*CDS2 with N-terminal Myc and C-terminal HA tags as well as *Tg*PIS with C-terminal HA tag (Δ*ku80*-TaTi strain)		
*Tg*CDS1-Myc-F (*BspH*I) *Tg*CDS1-HA-R (*Pac*I)	CTCATCTCATGAGTGAACAGAAGTTGATTTCCGAAGAAGACC TCGAACCCCCGTCGTCGC CTCATCTTAATTAATCAAGCGTAATCTGGAACATCGTATGGG TACCGAAATTCGGTTTGTCCTTGCAC	*pTETO7SAG1-UPKO* (Ectopic expression)
*Tg*CDS2-Myc-F (*BspH*I) *Tg*CDS2-HA-R (*Pac*I)	CTCATCTCATGAGTGAACAGAAGTTGATTTCCGAAGAAGACC TCGAGGAAACGCAGACTTCAGAGAAGC CTCATCTTAATTAATCAAGCGTAATCTGGAACATCGTATGGG TAGGCCTGCATCTGCGGGAC	
*Tg*PIS-F (*Nco*I) *Tg*PIS-HA-R (*Pac*I)	CTCATCCCATGGCGGGGACTTCTGCAAGCCG CTCATCTTAATTAATCAAGCGTAATCTGGAACATCGTATGGG TACGACGAGGGCGCACCAAA	
Making the Δ*tgcds1$_r$* mutant (Δ*ku80*-TaTi strain)		
*Tg*CDS1-F (*BspH*I) *Tg*CDS1-HA-R (*Pac*I)	CTCATCTCATGAAACCCCCGTCGTCGCCGATA CTCATCTTAATTAATCAAGCGTAATCTGGAACATCGTATGGG TACCGAAATTCGGTTTGTCCTTGCAC	*pTETO7SAG1-UPKO* (Ectopic expression)
*Tg*CDS1-5'UTR-F (*Xcm*I) *Tg*CDS1-5'UTR-R (*Spe*I)	CTCATCCCACCGGTCACCTGGCGAATCGTGTTTGTTTCCTCT T CTCATCACTAGTTCCGGGGTTTTGACCCTTA	*pTKO-DHFR-TS* (Homologous recombination)
*Tg*CDS1-3'UTR-F (*Hind*III) *Tg*CDS1-3'UTR-R (*Apa*I)	CTCATCAAGCTTCATTTGTAAGAGTATGCACACGTG CTCATCGGGCCCGGGAGCCTTGGTAGAGGTC	
*Tg*CDS1-5'Scr-F *Tg*CDS1-5'Scr-R	TGTCTTTCCTGCGTTGTCTG ACAGTCTCACCTCGCCTTGT	*pDrive* (Verification)
*Tg*CDS1-3'Scr-F *Tg*CDS1-3'Scr-R	CTCGCTGGTAGTCCCAACTG CAAAGCGAGACAGCATACCA	
Making the Δ*tgcds1$_r$*/Δ*tgcds2* mutant (Δ*tgcds1$_r$* mutant)		
*Tg*CDS2-5'UTR-F (*Apa*I) *Tg*CDS2-5'UTR-R (*Apa*I)	CTCATCGGGCCCAGTCCTGTCGATTTTCTCCCTG CTCATCGGGCCCTTTCGTCGCCTGCAAGTTT	*pTUB8-CAT* (Homologous recombination)
*Tg*CDS2-3'UTR-F (*Xho*I) *Tg*CDS2-3'UTR-R (*Xba*I)	CTCATCCTCGAGACGCAGGAGGGGGAGG CTCATCTCTAGAAGACGCTTCTTGCTCGCG	
*Tg*CDS2-5'Scr-F	AAGGCGACGAAACTTCAAC	*pDrive*

*Tg*CDS2-5'Scr-R	GCTTATCGATACCGTCGAGG	(Verification)
*Tg*CDS2-3'Scr-F *Tg*CDS2-3'Scr-R	CTATCAGTTGTTTTAGTCGAACCG CGAATCTGAATCTGAAGAAGACC	
Real-time PCR (Δ*tgcds1*$_r$ and Δ*tgcds1*$_f$/Δ*tgcds2* mutants)		
*Tg*CDS1-qPCR-F *Tg*CDS1-qPCR-R	GTCTCGTCACGTACACTGCG GATCACGAGCGAGGTTGG	Real-time PCR
*Tg*CDS2-qPCR-F *Tg*CDS2-qPCR-R	GCATCTTCAATCCTTCTCTCAA CCAAATCAGGGAGAAGATGG	
*Tg*EFA-qPCR-F *Tg*EFA-qPCR-R	AGTCGACCACTACCGGACAC CTCGGCCTTCAGTTTATCCA	
*Tg*TubA-qPCR-F *Tg*TubA-qPCR-R	AGGATGCTGCGAACAACTTC TCAAGAAACCCTGGAGACCA	
*Tg*GT1-qPCR-F *Tg*GT1-qPCR-R	GGCTATTTTGGCACCTTTCA AACGGGAAGACAAACCACAG	

2.1.5. Chemical reagents

1,4-Dithiothreitol (DTT)	Carl Roth (Karlsruhe, Germany)
5-Fluoro-2'-deoxyuridine (FUDR)	Sigma-Aldrich (St. Louis, MO)
ATP	Sigma-Aldrich (St. Louis, MO)
Agarose	Biozym (Hessisch Oldendorf, Germany)
Ampicillin	Applichem (Darmstadt, Germany)
Anhydrotetracycline (ATc)	IBA Lifesciences (Goettingen, Germany)
Bovine serum albumin fraction V (BSA)	Applichem (Darmstadt, Germany)
Bromophenol blue	Merck (Darmstadt, Germany)
Chloramphenicol	Carl Roth (Karlsruhe, Germany)
Chloroform	Carl Roth (Karlsruhe, Germany)
Crystal violet	Sigma-Aldrich (St. Louis, MO)
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich (St. Louis, MO)
DNA ladder (1 kb)	Fermentas (Waltham, MA)
Distilled water (HPLC-purified)	Carl Roth (Karlsruhe, Germany)
dNTP-Mix (100 mM)	Fermentas (Waltham, MA)
Dulbecco's modified eagle media (DMEM)	Biowest (Riverside, MO)
Ethanol	Carl Roth (Karlsruhe, Germany)
Fetal bovine serum (FBS)	PAN-Biotech (Aidenbach, Germany)
Fluoromount-G/DAPI	SouthernBiotech (Birmingham, AL)
Glycerol	Applichem (Darmstadt, Germany)
Hank's balanced salt solution (HBSS)	PAA (Linz, Austria)
Isopropanol	Applichem (Darmstadt, Germany)

L-glutamine (100x stock)	Biowest (Riverside, MO)
MEM non-essential amino acids (100x stock)	Biowest (Riverside, MO)
Methanol	Carl Roth (Karlsruhe, Germany)
Mycophenolic acid (MPA)	Applichem (Darmstadt, Germany)
Natural and synthetic lipids	Avanti Polar Lipids (Alabaster, AL)
Paraformaldehyde	Merck (Darmstadt, Germany)
Penicillin/Streptomycin (100x stock)	Biowest (Riverside, MO)
Phosphat buffered saline (PBS)	Biowest (Riverside, MO)
Protein marker (prestained)	Fermentas (Waltham, MA)
Pyrimethamine	AK Scientific (Union City, CA)
Rotiphorese gel 30 (Acrylamide)	Carl Roth (Karlsruhe, Germany)
Sodium pyruvate (100x stock)	Biowest (Riverside, MO)
Tris-HCl	Promega (Fitchburg, WI)
Trizol	Life Technologies (Waltham, MA)
Trypsin/EDTA	Biowest (Riverside, MO)

Other standard chemicals used in preparations for media and buffers were purchased from AppliChem, Carl Roth or Sigma-Aldrich.

2.1.6. Buffers and media

T. gondii culture - Cytomix

120 mM KCl; 25 mM HEPES (pH 7.6); 5 mM $MgCl_2$; 2 mM EDTA; 0.15 mM $CaCl_2$; 10 mM K_2HPO_4/KH_2PO_4 (pH 7.6); in deionized H_2O; adjusted to pH 7.6; filter-sterilized with 0.22 µm filter; stored at 4 °C.

T. gondii culture - D10 medium

500 ml DMEM (high glucose, no L-glutamine); 50 ml iFBS (heat-inactivated); 5.5 ml L-glutamine (200 mM); 5.5 ml non-essential amino acids (100x); 5.5 ml penicillin/streptomycin (100x); 5.5 ml sodium pyruvate (100 mM); stored at 4 °C.

T. gondii culture - Freezing stock medium

10% DMSO in heat-inactivated iFBS, stored at -20 °C.

E. coli culture - LB medium

10 g tryptone; 5 g yeast extract; 10 g NaCl; optional 15 g agar for plates; in 1 L deionized H_2O; autoclaved.

E. coli culture - SOB/SOC medium

20 g tryptone; 5 g yeast extract; 0.5 g NaCl; 0.186 g KCl; in 1 L deionized H_2O; autoclaved; 0.952 g $MgCl_2$; optional 3.603 g glucose for SOC medium; filter-sterilized with 0.22 µm filter.

Preparation of competent *E. coli* - TFB-I buffer

30 mM KOAc; 50 mM $MnCl_2$; 100 mM RbCl; 10 mM $CaCl_2$; 15% Glycerol; adjusted to pH 5.8; filter-sterilized with 0.22 µm filter; stored at 4 °C.

Preparation of competent *E. coli* - TFB-II buffer

10 mM NaMOPS; 10 mM RbCl; 75 mM $CaCl_2$; 15% Glycerol; adjusted to pH 7.0; filter-sterilized with 0.22 µm filter; stored at 4 °C.

Agarose electrophoresis - TAE buffer

40 mM Tris-HCl (pH8); 1 mM EDTA; 0.11% acetic acid; in deionized H_2O.

Western blotting - 5% stacking gel

1.4 ml deionized H_2O; 0.33 ml 30% acrylamide; 0.25 ml 1 M Tris-HCl (pH 6.8); 20 µl 10% SDS; 20 µl 10% APS; 10 µl TEMED.

Western blotting - 10% resolving gel

1.93 ml deionized H_2O; 1.67 ml 30% acrylamide; 1.3 ml 1.5 M Tris-HCl (pH 8.8); 50 µl 10% SDS; 50 µl 10% APS; 3 µl TEMED.

Western blotting - SDS loading buffer (2x)

0.1% bromophenol blue; 100 mM DTT; 20% glycerol; 5% mercaptoethanol; 4% SDS; 100 mM Tris-HCl (pH 6.8); in deionized H_2O.

Western blotting - SDS running buffer (5x)

1.25 M glycine; 0.5% SDS; 125 mM Tris-HCl (pH 8.3); in deionized H_2O.

Western blotting - Semi-dry blot transfer buffer

38 mM glycine; 20% methanol; 0.0037% SDS; 48 mM Tris-HCl (pH 8.3); in deionized H_2O.

Western blotting - TBS buffer (10x)
0.5 M Tris-HCl (pH 7.6); 1.5 M NaCl; in deionized H_2O.

Western blotting - TBS-T buffer
10% 10x TBS buffer; 0.2% Tween 20; in deionized H_2O.

Western blotting - Blocking buffer
5% skimmed milk powder; in TBS-T buffer.

2.1.7. Enzymes

Antarctic phosphatase	New England Biolabs (Ipswich, MA)
Dream Taq polymerase	Fermentas (Waltham, MA)
Klenow enzyme	New England Biolabs (Ipswich, MA)
Pfu Ultra II Fusion HS DNA polymerase	Stratagene (Heidelberg, Germany)
Proteinase K	Sigma-Aldrich (St. Louis, MO)
Q5 polymerase	New England Biolabs (Ipswich, MA)
Restriction endonucleases	New England Biolabs (Ipswich, MA)
T4 ligase	Life Technologies (Waltham, MA)

2.1.8. Commercial kits

CloneEZ PCR cloning kit	GenScript (Piscataway, NJ)
First-strand cDNA synthesis kit	Life Technologies (Waltham, MA)
Genomic DNA preparation kit	Analytik Jena (Jena, Germany)
InnuPREP DOUBLEpure kit	Analytik Jena (Jena, Germany)
InnuPREP plasmid mini kit	Analytik Jena (Jena, Germany)
pDrive PCR cloning kit	Qiagen (Hilden, Germany)
Platinum SYBR green qPCR superscript-UDG kit	Invitrogen (Waltham, MA)
PureLink RNA mini kit	Life Technologies (Waltham, MA)
PureLink HiPure plasmid midiprep kit	Life Technologies (Waltham, MA)

2.1.9. Instruments

Camera system (E.A.S.Y. RH)	Herolab (Wiesloch, Germany)
Centrifuge (5415C, 5417R and 5810R)	Eppendorf (Hamburg, Germany)
Centrifuge (Ultra, Avanti J-26S XP)	Beckmann Coulter (Brea, CA)

Counting chamber (Neubauer improved)	Fuchs Rosenthal (Freiburg, Germany)
Cryo container (Nalgene Mr. Frosty)	Thermo Fisher Scientific (Waltham, MA)
Electric pipetting aid (Accu-jet Pro)	Brand (Wertheim, Germany)
Electrophoresis power supply (EPS 300)	Pharmacia Biotech (Uppsala, Sweden)
Electroporator (Amaxa Nucleofector)	Lonza (Basel, Switzerland)
Electroporator (BTX Square Porator ECM 830)	BTX (Holliston, MA)
Gel electrophoresis system (Easy Phor)	Biozym (Hessisch Oldendorf, Germany)
Ice machine (ZBE 110-35)	Ziegra (Isernhagen, Germany)
Incubator (Modell 500)	Memmert (Schwabach, Germany)
Incubator-CO_2 (APT.line C 150)	Binder (Tuttlingen, Germany)
Incubator-CO_2 (FUNCTIONLine)	Heraeus Instruments (Hanau, Germany)
Incubator-CO_2 (HERACELL 150i)	Thermo Fisher Scientific (Waltham, MA)
Incubator-shaking (New Brunswick Innova 4000)	Eppendorf (Hamburg, Germany)
Infrared imaging system (Odyssey Fc)	LI-COR Biosciences (Lincoln, NE)
Microscope-fluorescence (Axio Image.Z2)	Zeiss (Oberkochen, Germany)
Microscope-inverted (LABOVERT)	Leica (Wetzlar, Germany)
Microscope-light optical (DM750)	Leica (Wetzlar, Germany)
Microwave (M805 Typ KOR-6115)	Alaska (Düsseldorf, Germany)
PCR cycler (Flex Cycler)	Analytik Jena (Jena, Germany)
Photometer (BioPhotometer)	Eppendorf (Hamburg, Germany)
Photometer (NanoDrop spectral, ND-1000)	Peqlab (Darmstadt, Germany)
Pipette	Eppendorf (Hamburg, Germany)
Pipette-multichannel (Transferpipette-8/-12)	Brand (Wertheim, Germany)
Real-time RT-PCR cycler (Mastercycler realplex^2)	Eppendorf (Hamburg, Germany)
Safety work bench (HeraSafe)	Heraeus Instruments (Hanau, Germany)
Scale (BP 110 S)	Sartorius (Göttingen, Germany)
Scale (FA-30100-2)	Faust (Schaffhausen, Switzerland)
Scale (PCB400-2)	Kern & Sohn (Balingen, Germany)
SDS-PAGE system (SE 250 Mighty Small II)	Hoefer (Holliston, MA)
Shaker-thermo (Thermomixer comfort)	Eppendorf (Hamburg, Germany)
Steam-sterilizer (VARIOKLAV)	Thermo Fisher Scientific (Waltham, MA)
UV-transilluminator (UVT-20M/W)	Herolab (Wiesloch, Germany)
Waterbath (U3)	Julabo (Seelbach, Germany)
Waterbath (WB-4MS)	Biosan (Riga, Latvia)

Western Blotting System (SEDEC M)	Peqlab (Darmstadt, Germany)

2.1.10. Plasticware and other disposables

Cell culture plate (6-, 24- and 96-well)	Sarstedt (Nümbrecht, Germany)
Cell culture dish (60x15 mm)	Sarstedt (Nümbrecht, Germany)
Cell scraper (30 cm)	TPP (Trasadingen, Switzerland)
Cuvette-electroporation	Eppendorf (Hamburg, Germany)
Cuvette-UV	Carl Roth (Karlsruhe, Germany)
DuraSeal laboratory sealing film	Diversified Biotech (Dedham, MA)
Filter (5 µm)	Merck Millipore (Billerica, MA)
Filter (0.22 µm)	Schleicher Schuell (München, Germany)
Glass cover slip and microscopic slide	Carl Roth (Karlsruhe, Germany)
Glove	Sempermed (Vienna, Austria)
Hypodermic needle	BD Bioscience (Franklin Lakes, NJ)
Nitrocellulose transfer membrane	Applichem (Darmstadt, Germany)
Parafilm	Bemis Company (Neenah, WI)
Petri dish (94x16 mm)	Greiner Bio One (Kremsmünster, Austria)
Pipette-pasteur	A. Hartenstein (Würzburg, Germany)
Pipette-serological (10 ml and 25 ml)	Greiner Bio One (Kremsmünster, Austria)
Syringe	BD Bioscience (Franklin Lakes, NJ)
Syringe-hamilton	Hamilton (Reno, NV)
Tip-pipette (10 - 1000 µl)	Greiner Bio One (Kremsmünster, Austria)
Tip-RNAase free barrier (10 - 1000 µl)	Biozym (Hessisch Oldendorf, Germany)
Tube-borosilicate glass (16 ml)	Sigma-Aldrich (St. Louis, MO)
Tube-cryo preservation (1.8 ml)	Sarstedt (Nümbrecht, Germany)
Tube-falcon (15 ml and 50 ml)	Greiner Bio One (Kremsmünster, Austria)
Tube-PCR (0.2 ml))	Sarstedt (Nümbrecht, Germany)
Tube-PCR stripe (0.2 ml)	Biozym (Hessisch Oldendorf, Germany)
Tube-polypropylene culture (12 ml)	Greiner Bio One (Kremsmünster, Austria)
Tube-reaction (1.5 ml and 2 ml)	Sarstedt (Nümbrecht, Germany)
Whatman paper (3 MM)	A. Hartenstein (Würzburg, Germany)

2.2. Bioinformatics and phylogeny studies

Initial identification of the enzymes in phospholipid biogenesis pathways of protozoan parasites was performed using EuPathDB (www.eupathdb.org) and ToxoDB (www.toxodb.org) [33,77]. The functional domains and transmembrane regions of the enzymes were predicted by Simple Modular Architecture Research Tool (SMART) (http://smart.embl-heidelberg.de) and Transmembrane Hidden Markov Model (TMHMM) (http://www.cbs.dtu.dk/services/TMHMM/). The signal and transit peptides were predicted by SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP/), ChloroP 1.1 (http://www.cbs.dtu.dk/services/ChloroP/) and PlasmoAP algorithm (http://v4-4.plasmodb.org/restricted/PlasmoAPcgi.shtml). The peptides for mitochondrial targeting were predicted by Mitoprot (https://ihg.gsf.de/ihg/mitoprot.html). The phylogenetic trees were constructed with CLC Sequence Viewer 7.7 (http://www.clcbio.com/products/clc-sequence-viewer/) and visualized with FigTree v1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/).

2.3. Parasite and host cell cultivation

2.3.1. Propagation of *E. falciformis* in mice and isolation of sporozoites

The life cycle of *E. falciformis* was maintained by continuous passage of parasite oocysts in NMRI mice [25]. Oocysts in the animal feces were washed in water, sterilized, floated with NaOCl, and stored in potassium dichromate at 4 °C up to 3 months [18]. Purified oocysts were digested with 0.4% pepsin (pH 3, 37 °C, 1 h) before wash with PBS. Sporocysts were released by mixing oocyst pellet with glass beads (0.5 mm) and vortexing, and incubated in DMEM medium supplemented with 0.25% trypsin, 0.75% sodium tauroglycocholate, 20 mM glutamine, 100 units/ml penicillin and 100 ug/ml streptomycin at 37 °C for 2 h. Free sporozoites were column-purified by DE-52 anion exchange chromatography [78] and stored at -80 °C for lipid analysis.

2.3.2. Propagation of HFF cells

HFFs were cultured in D10 medium at 37 °C with 5% CO_2 in a humidified incubator. The cells were harvested by trypsinization and grown to confluence in cell culture flasks, dishes or plates, as required.

2.3.3. *In vitro* culture of *T. gondii* and purification of tachyzoites

Tachyzoites of *T. gondii* were propagated by serial passage in HFF monolayers at a MOI of 3. For all assays, parasites were mechanically released from late-stage cultures and used immediately. Parasitized cells (40-42 h post infection) were scraped in fresh culture medium and squirted through 23G and 27G syringes (2x each) to obtain extracellular tachyzoites for direct use in transfection and lytic cycle assays, For lipidomics study, syringe-released parasites were additionally filtered by 5 µm filters and stored in -80 °C. For quantification, parasites were diluted appropriately and counted using the Neubauer improved counting chamber.

2.4. Molecular cloning and construction of vectors

2.4.1. Preparation of parasite RNA and DNA

Parasite RNA was isolated from freshly purified *E. falciformis* sporozoites or syringe-released *T. gondii* tachyzoites using TRIzol-based extraction method with PureLink RNA mini kit and subsequently reverse-transcribed using First-strand cDNA synthesis kit with oligo-dT primer. Parasite gDNA was isolated using the genomic DNA preparation kit. RNA, cDNA and gDNA samples were stored at -80 °C, -20 °C and 4 °C, respectively.

2.4.2. PCR reactions

All DNA amplicons for molecular cloning were amplified using Pfu Ultra II Fusion polymerase or Q5 polymerase. 10-200 ng of cDNA or gDNA was used as template in standard PCR reactions in a Flex PCR according to the manufacturer's protocol. For colony PCR, *E. coli* cells were suspended in 20 µl of distilled H_2O, and 3 µl was used as PCR template with Dream Taq polymerase. The PCR outcome was mixed with DNA-loading dye before DNA was separated on 0.8 % agarose gels stained with RedSafe DNA staining solution at 90-120 V in TAE buffer and visualized by a UV-transilluminator. PCR products were purified from column or agarose gel using the innuPREP DOUBLEpure kit. NanoDrop Spectralphotometer ND-1000 was used to determine the DNA concentration.

2.4.3. DNA digestion and ligation

DNA fragments were cloned into the respective vectors either by ligation-independent or restriction endonuclease-mediated cloning. CloneEZ PCR cloning kit was used for ligation-independent cloning. For restriction cloning, DNA fragments and vectors were digested with 3 units per µg DNA of indicated restriction endonucleases following manufacturing instruction. Digested insert and vector DNA was purified and ligated in a molar ratio of 3:1 or 5:1 (fmol of insert : fmol of vector) for sticky or blunt end ligation, respectively, using T4 ligase at room temperature for 1 h or at 4 °C overnight before transformation in *E. coli*.

2.4.4. Preparation of competent *E. coli* cells

5 ml SOB culture (with 12.5 µg/ml tetracycline) of XL-1Blue cells was inoculated over night, then diluted in 200 ml of the same medium for further inoculation at 37 °C until OD_{600} reaches 0.4 - 0.6. Cells were harvested (1300 *g*, 10 min, 4°C). The pellet was washed once in 50 ml of ice-cold TFB-I buffer and resuspended in 6.4 ml of TFB-II buffer before stocking at -80 °C.

2.4.5. Transformation of *E. coli*

The ligation reaction was mixed with competent *E. coli* XL-1Blue cells (90-100 µl) before incubation for 30 min on ice. The cells were then heat-shocked for 45 sec at 42 °C and immediately chilled on ice for 2 min before addition of 700 µl SOC medium and incubation at 37 °C for 1 h. Cells were pelleted and suspended in 100 µl of fresh SOC medium for plating on selective LB-agar containing 0.1 mg/ml ampicillin. Plates were incubated at 37 °C overnight and colonies were screened for plasmid expression by colony PCR. Positive clones were selected for plasmid isolation and stocking by cryo-preserving overnight culture in 25% glycerol at -80 °C.

2.4.6. Isolation of plasmid DNA

For plasmid DNA preparation, 5 ml of *E. coli* overnight culture was used with the innuPREP plasmid mini kit. For preparation of large-scale plasmid, 200 ml of *E. coli* overnight culture was used with the PureLink HiPure plasmid midiprep kit. Plasmid samples were stored at -20 °C.

2.5. Generation of transgenic *T. gondii* tachyzoites

2.5.1. Transfection of tachyzoites and making of clonal transgenic lines

The respective plasmid constructs were transfected into freshly released tachyzoites of specified strains suspended in cytomix using a BTX 830 electroporator (50 µg plasmid DNA, ~10^7 parasites, 700 µl cytomix, 30 µl 100 mM ATP, 12 µl 250 mM GSH, 2 kV, 50 Ω, 25 µF, 250 µs) or an Amaxa Nucleofector (10 µg plasmid DNA, ~2x10^6 parasites, 100 µl cytomix, 2 µl 100 mM ATP, 2 µl 250 mM GSH, program T-16). Transformed tachyzoites were used to infect HFF cells immediately and selected for resistance to a drug corresponding to the selection marker encoded by transfected plasmid. The drug-resistant transgenic parasites were cloned by limiting dilution in 96-well plates and individual clones were screened by PCR and/or immunofluorescence assays. Positive stable transgenic lines were selected for further experiments.

2.5.2. Generation of tachyzoites expressing *E. falciformis* enzymes

For ectopic expression of selected *E. falciformis* enzymes in *T. gondii*, all enzyme ORFs were cloned into the *pGRA1-UPKO* vector at *Nsi*I/*Pac*I restriction sites (Appendix 8A). Gene-specific primers used for PCRs are listed in Table 1. Constructs were transfected into freshly released tachyzoites of the RHΔ*ku80*-Δ*hxgprt* strain. Parasites expressing ER-localized enzymes (*Ef*G3PAT1, *Ef*LPAAT2, *Ef*CDS1 and *Ef*PTS) and Golgi-localized enzymes (*Ef*LPAAT1, *Ef*PSS, *Ef*CEPT1, *Ef*CEPT2 and *Ef*PIS) were co-transfected with the constructs encoding *Tg*PTS-Myc and *Tg*ERD2-Ty1 for co-localization studies. The transfected parasites were used to infect HFFs for immunofluorescence assays and screened for positive transient expressions of *E. falciformis* enzymes.

2.5.3. Generation of the Δ*tgpts*/*Ef*PTS strain

The *pGRA1-UPKO-EfPTS* construct (generated as described above, Appendix 8A) was transfected into the Δ*tgpts* strain. Transfected tachyzoites were selected for the disruption of the uracil phosphoribosyltransferase (*UPRT*) locus using 5 µM FUDR [36]. The drug-resistant transgenic parasites were cloned and positive clones were used for the lytic cycle assays and lipidomics studies.

2.5.4. Generation of transgenic tachyzoites for localization study of selected *T. gondii* enzymes

For tagging of the *Tg*CDS1, *Tg*CDS2 and *Tg*PGPS genes with a C-terminal HA tag, 1.0 to 1.3 kb of the 3'-end of these genes excluding stop codon (crossover sequence, COS) were amplified using tachyzoite gDNA and gene-specific primers (Table 1). Amplicons were inserted into the *pG152* vector by ligation independent cloning (Appendix 8B-D). Constructs were linearized using an appropriate enzyme (*Nae*I, *Xho*I or *Sac*I as specified in figures) present in the COS and transfected into the RHΔ*ku80*-Δ*hxgprt* strain. Parasites were selected for hypoxanthine-xanthine-guanine phosphoribosyltransferase (HXGPRT) expression using 25 µg/ml MPA and 50 µg/ml xanthine [37]. The resulting transgenic strains expressed *Tg*CDS1-HA, *Tg*CDS2-HA or *Tg*PGPS-HA under the control of corresponding endogenous promoters and 3'UTR of *Tg*SAG1. Parasites expressing *Tg*CDS1-HA were subsequently transfected with the construct *pTUB8-TgDER1-GFP* for co-localization studies.

For ectopic expression of *Tg*CDS1$^{398\text{-}1068}$-Myc (lacking N-terminal extension and tagged with a C-terminal Myc epitope), the partial ORF was cloned into the *pTKO-CAT* plasmid at *Nsi*I/*Pac*I sites (Appendix 8E). The deletion variants of *Tg*CDS2, including *Tg*CDS2$^{527\text{-}1044}$-Myc and those lacking the signal peptide (*Tg*CDS2$_{\Delta SP}$-Myc), transit peptide (*Tg*CDS2$_{\Delta TP}$-Myc) or the entire bipartite sequence (*Tg*CDS2$_{\Delta BS}$-Myc), were engineered in the *pTKO-DHFR-TS* vector using appropriate primers at *Nsi*I/*Pac*I sites (Appendix 8F). The plasmid constructs were linearized with *Not*I and transfected into strains expressing full-length *Tg*CDS1-HA or *Tg*CDS2-HA. For ectopic expression of *Tg*CDS1 and *Tg*CDS2 with dual epitopes (N-terminal Myc tag and C-terminal HA tag), as well as *Tg*PIS with a C-terminal HA tag, their cDNAs were ligated into the *pTETO7SAG1-UPKO* plasmid at *Nco*I/*Pac*I sites (Appendix 8G-I). Constructs were linearized by *Not*I and then transfected into the RHΔ*ku80*-TaTi strain. Parasites expressing *Tg*PIS-HA were subsequently transfected with a construct encoding for *Tg*ERD2-Ty1 (regulated by p*GRA1* promoter) for co-localization studies.

2.5.5. Generation of the Δ*tgcds1$_r$* and Δ*tgcds1$_r$*/Δ*tgcds2* mutants

The conditional mutant of *Tg*CDS1 (Δ*tgcds1$_r$*) was generated in two steps. First, *Tg*CDS1-HA regulated by ATc-repressible promoter (p*TETO7SAG1*) was targeted at the *TgUPRT* locus. The ORF of *Tg*CDS1 containing a C-terminal HA tag was inserted into the *pTETO7SAG1-UPKO* vector at *Nco*I/*Pac*I restriction sites (Appendix 8J). The eventual construct was linearized by *Not*I and transfected into the RHΔ*ku80*-TaTi strain, followed by negative selection for the disruption of the *TgUPRT* locus using 5 μM FUDR [36]. In the second step, the *TgCDS1* locus was deleted by double homologous recombination in the merodiploid strain expressing an ATc-regulatable copy of *Tg*CDS1. To achieve this, the 5' and 3'UTRs of *Tg*CDS1 were amplified from tachyzoite gDNA and cloned into the *pTKO-DHFR-TS* vector using *Xcm*I/*Spe*I and *Hind*III/*Apa*I enzyme pairs, respectively (Appendix 8K). The construct was linearized using *Apa*I, and transfected into the merodiploid strain generated in the first step. The conditional mutant was selected for the expression of dihydrofolate reductase-thymidylate synthase (DHFR-TS) using 1 μM pyrimethamine [34]. To make a knockout of the *Tg*CDS2 gene, the 5' and 3'UTRs amplified from tachyzoite gDNA were cloned into the *pTUB8-CAT* plasmid at *Apa*I and *Xho*I/*Xba*I restriction sites, respectively (Appendix 8L). The plasmid was linearized (*Xba*I) and transfected into Δ*tgcds1$_r$* strain, followed by selection for the expression of chloramphenicol acetyltransferase (CAT) using 20 μM chloramphenicol [35]. The resulting strain (Δ*tgcds1$_r$*/Δ*tgcds2*) lacked the expression of *Tg*CDS2 entirely and allowed conditional knockdown of *Tg*CDS1 by ATc treatment.

2.6. RNA and protein detection

2.6.1. Real-time PCR

Total RNA was first reverse-transcribed using oligo-dT primer and analyzed by SYBR green-based assays in a Mastercycler. The relative expression of transcripts (fold-induction) was calculated with respect to the parental strain using the ΔΔ*CT* method. Transcripts of elongation factor A (EFA), tubulin A (TubA) and glucose transporter 1 (GT1) were used as housekeeping genes to normalize the expression of *Tg*CDS1 and *Tg*CDS2 across samples.

2.6.2. Indirect immunofluorescence assay

Parasitized HFFs cultured on glass coverslips were washed with PBS 24 h post-infection, fixed with 4% paraformaldehyde for 10 min, and neutralized with 0.1 M glycine in PBS for 5 min. Cells were permeabilized with 0.2% triton X-100 in PBS for 20 min and treated with 2% BSA in 0.2% triton X-100 in PBS for 30 min. Samples were stained with a combination of primary antibodies for 1 h, as shown in respective figures. Cells were washed three times with 0.2% triton X-100 in PBS and then stained with Alexa488/594-conjugated antibodies for 45 min. Following three additional washings with PBS, samples were mounted in fluoromount-G/DAPI and stored at 4 °C. Imaging was done using a fluorescence microscope. For co-localization studies, the organelle markers used in this study include *Tg*PTS-Myc and TgDER1-GFP for ER, *Tg*Fd for apicoplast, *Tg*ERD2-Ty1 for Golgi body, *Tg*F1B for mitochondrion, *Tg*GAP45 for parasite periphery, as well as *Tg*GRA1 for DG and PV.

2.6.3. Immunoblot assay

Fresh extracellular parasites ($1.5x10^7$) were washed twice with PBS, pelleted (400 *g*, 10 min, 4°C), resuspended in loading buffer and subjected to gel electrophoresis. Proteins were resolved by 10% SDS-PAGE and then transferred to a nitrocellulose membrane (85 mA, 90 min). The membrane was treated with 5% skimmed milk suspended in TBS-T buffer (overnight, 4 °C), incubated with anti-HA and anti-*Tg*HSP90 antibodies (2 h at room temperature), washed 3 times for 5 min each, and incubated with IRDye-conjugated secondary antibodies 680RD and 800CW. Proteins were visualized using LI-COR imaging system.

2.7. Lytic cycle assays

For assays with the Δ*tgcds1*$_r$ and Δ*tgcds1*$_r$/Δ*tgcds2* mutants, tachyzoites were pretreated with or without ATc (1 µM) for 2 passages (4 d) before assays, as well as during the assays.

2.7.1. Plaque assay

Plaque assays were performed by infecting HFF monolayers in 6-well plates (250 tachyzoites per well). In assays with Δ*tgcds1*$_r$ and Δ*tgcds1*$_r$/Δ*tgcds2* mutants, CDP-DAG, PtdIns, PtdGro and cardiolipin, were dissolved in serum and added to plaque cultures (0.05-0.1 µM). Cultures were incubated unperturbedly for 7 days and

samples were fixed with ice-cold methanol for 2 min and stained with crystal violet dye for 15 min. Plaques were imaged and scored for sizes and numbers using the ImageJ software.

2.7.2. Yield assay

$3x10^6$ tachyzoites of each strain were used to infect confluent HFFs (MOI, 3). Parasites were syringe-released from host cells after 40 h and numerated.

2.7.3. Invasion assay

$1x10^7$ tachyzoites of each strain were used to infect confluent HFFs (MOI, 10) for 1 h and fixed. Noninvasive parasites were stained with anti-*Tg*SAG1 antibody prior to detergent permeabilization. Cells were then washed 3 times with PBS, permeabilized with 0.2% triton 100/PBS for 20 min, and stained with anti-*Tg*GAP45 antibody to visualize intracellular parasites. Samples were then treated with anti-mouse IgG Alexa Fluor 488 and anti-rabbit IgG Alexa Fluor 594 antibodies before washing and mounting in fluoromount-G/DAPI. Intracellular and extracellular parasites were distinguished by differential staining with *Tg*GAP45 signal only or with both *Tg*SAG1 and *Tg*GAP45 signals, respectively. The percentages of invaded parasites were used to compare the invasion efficiencies of the strains.

2.7.4. Replication and egress assays

Confluent HFFs cultured on coverslips in 24-well plates were infected with tachyzoites of each strain (MOI, 1), fixed at indicated time points (40 h for replication assays; 48 and 72 h for egress assays), and then subjected to immunostaining using anti-*Tg*GAP45 antibody. For replication assays, the mean percentages of vacuoles containing variable numbers of intracellular parasites were scored to examine the replication phenotype. For egress assays, the rate of egressed vacuoles was calculated by comparing the vacuole numbers between 48 h and 72 h.

2.7.5. Motility assay

For motility assays, freshly syringe-released parasites were incubated on BSA (0.01%)-coated coverslips in HBSS buffer (15 min, 37°C), fixed with 4% paraformaldehyde and 0.05% glutaraldehyde (10 min), and then stained with anti-

*Tg*SAG1 and Alexa488 antibodies. Motile fractions and trail lengths were quantified using the ImageJ software.

2.8. Lipid analysis

Pellets of *E. falciformis* sporozoites and *T. gondii* tachyzoites ($3x10^7$ parasites per sample) were suspended in 0.8 ml PBS and lipids were extracted according to Bligh-Dyer method [79]. Briefly, 1 ml chloroform and 2 ml methanol were added before vortexing and incubating for 20 min. Then samples were centrifuged (2000 *g*, 5 min), transferred to a new glass tube (only supernatant) and mixed with 2 ml water and 2 ml chloroform. After votexing and centrifuging (2000 *g*, 5 min), the upper chloroform phase was transferred to a conical glass tube. The lower phase was mixed with 2 ml chloroform, vertexed and centrifuged (2000 *g*, 5 min) before the upper phase was transferred to the previous conical tube. Lipids were dried under nitrogen and suspended in 1 ml of chloroform and methanol 1:1 mixture, from which 10 µl aliquot was introduced onto a Kinetex HILIC column (dimensions 50x4.6 mm, 2.6 µm, Phenomenex, Torrance CA). Phospholipids were resolved at a flow rate of 1 ml/min, as described elsewhere [80]. The column effluent was introduced into a mass spectrometer instrument (LTQ-XL, Thermo Scientific, Waltham, MA) and analyzed by electrospray ionization in positive and negative ion modes. Calibration curves of authentic standards were used to quantify lipids. Fatty acid composition of individual lipid was determined by MS/MS. Data were processed using the package "XCMS" in R (https://www.R-project.org/) [81].

2.9. Virulence assay in mice

Tachyzoites of the parental (RHΔ*ku80*-TaTi) and Δ*tgcds1*$_r$ strains were pretreated with or without ATc (1 µM) for 3 passages (6 d) in cultures. ATc treatment of C57BL/6J mice (female, 6-8 weeks old) was initiated 2 days before inoculation by supplying the drug in drinking water (0.2 mg/ml) and continued for 2 weeks during infection. Animals were inoculated with fresh extracellular tachyzoites (10^3) *via* intraperitoneal route and monitored for mortality and morbidity over a period of 4 weeks. For re-infection, animals immunized with the Δ*tgcds1*$_r$ strains (ATc-treated) were challenged with tachyzoites of the parental strains (10^3) and monitored for additional 28 days.

2.10. Statistics

All data are shown as mean with standard error of mean (SEM) from three or six independent assays as indicated in figure legends. Statistical analyses were performed using the GraphPad Prism program (Version 5). Significance was tested by unpaired two-tailed Student's *t* test with equal variances or ANOVA (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

3. Results

3.1. Exclusive phospholipid expression and autonomous membrane biogenesis in *E. falciformis* indicate a host-independent lifestyle of apicomplexan sporozoites

3.1.1. Lipid profile of *Eimeria* sporozoites differs markedly from *Toxoplasma* tachyzoites

To examine the phospholipid composition of *E. falciformis* sporozoites, we isolated total lipids from purified parasites and performed high-performance liquid chromatography (HPLC). The total lipids extracted from tachyzoites of *T. gondii*, which served as a comparative reference, were also analyzed alongside. The most abundant phospholipid detected in sporozoites was PtdCho (79.32%), followed by PtdEtn (13.12%), PtdThr (5.75%), PtdIns (1.26%), PtdSer (0.51%) and PtdGro (0.04%), whereas the sole dominant sphingolipid is inositol phosphorylceramide (IPC) (Figure 5A). *T. gondii* tachyzoites shared similar phospholipid classes with *E. falciformis* sporozoites (PtdCho 79.04%, PtdEtn 9.09%, PtdThr 5.89%, PtdIns 4.35%, PtdSer 1.55% and PtdGro 0.09%), but possessed ethanolamine phosphorylceramide (EPC) and sphingomyelin (SM) instead of IPC as the major sphingolipids, as also reported previously [45,50] (Figure 5A). The PtdThr peaks of *E. falciformis* and *T. gondii* appeared at different retention time, which indicated the presence of divergent lipid species. Quantification of lipids based on external calibration standard showed that *E. falciformis* sporozoites harbor more PtdCho, PtdEtn and PtdThr, however less PtdIns, PtdSer and PtdGro than *T. gondii* tachyzoites (Figure 5B).

Mass spectrometry (MS) analysis of HPLC-fractionated phospholipids revealed that the acyl-chain compositions of different species were much more uniform in sporozoites compared to tachyzoites. The most abundant species in each phospholipid class of tachyzoites included PtdCho 36:2 (22%), PtdEtn 36:3 and 34:2 (both 23%), PtdThr 40:5 (62%), PtdIns 34:1 (46%), PtdSer 36:3 (17%) and PtdGro 30:0 (45%) (Figure 5C). On the other hand, C36:2 (18:1/18:1) was the dominant species of most lipids in sporozoites, followed by shorter chains between C30 and C34 (Figure 5C). PtdThr was the only exception with C36:1 (41%) and C36:2 (26%) as its first and second most abundant species (Figure 5C). Moreover, when

compared to other sporozoite phospholipids, PtdThr and PtdSer contained a higher proportion of long acyl chains C38-C42 (26% of PtdThr and 20% of PtdSer) and less of shorter acyl chains C28-C34 (7% of PtdThr and 3% of PtdSer). Collectively, these results show the presence of notably distinct lipid species in otherwise closely related coccidian parasites, *E. falciformis* and *T. gondii*.

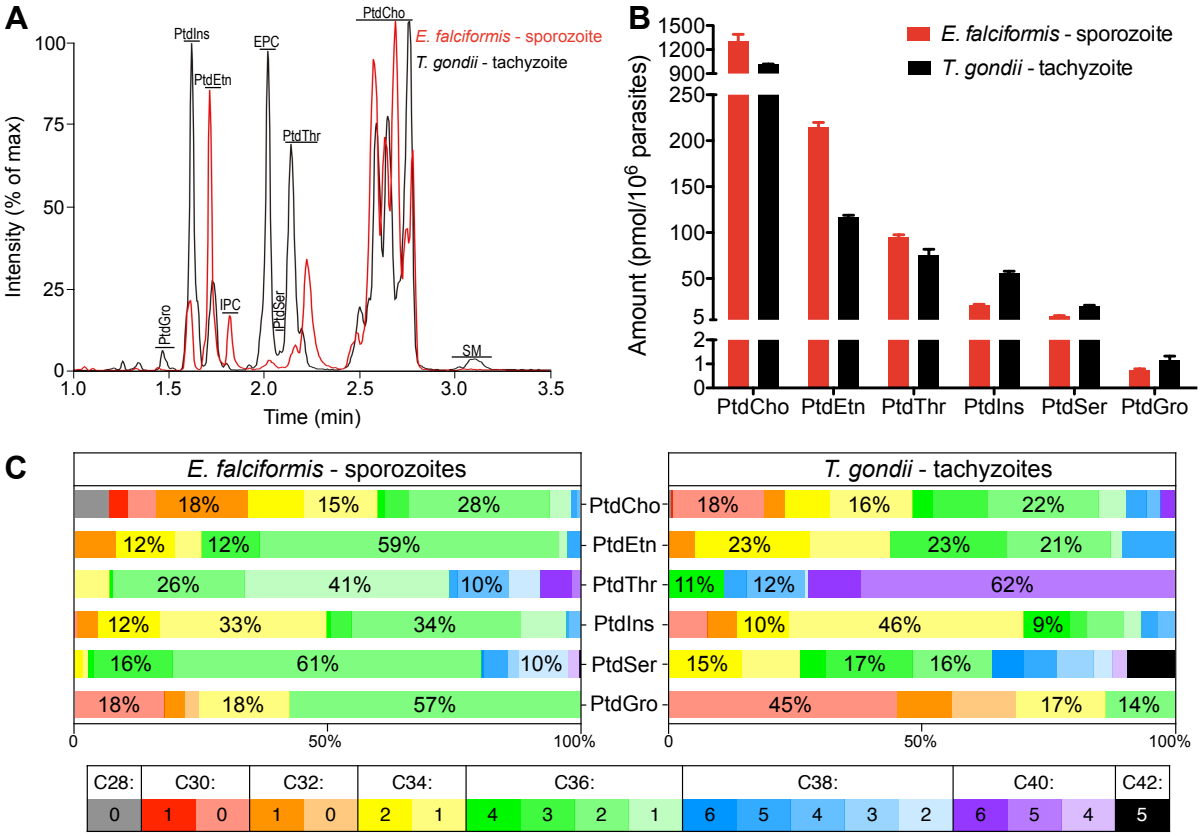


Figure 5. Lipidomics identifies differences and similarities of the phospholipid compositions between *E. falciformis* sporozoites and *T. gondii* tachyzoites. (**A**) Retention times and relative intensities of lipids isolated from *E. falciformis* sporozoites (red) and *T. gondii* tachyzoites (black). (**B**) Amounts of major phospholipids in *E. falciformis* (red) and *T. gondii* (black). (**C**) Colored columns comparing the compositions of major phospholipid species between the two parasites. Percentages of the three most abundant species in each lipid are shown on the corresponding columns. *Abbreviations*: EPC, ethanolamine phosphorylceramide; IPC, inositol phosphorylceramide; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; PtdGro, phosphatidylglycerol; PtdIns, phosphatidylinositol; PtdSer, phosphatidylserine; PtdThr, phosphatidylthreonine; SM, sphingomyelin.

3.1.2. *Eimeria* sporozoites encode entire network for *de novo* phospholipid biogenesis

Having identified major lipid classes expressed in sporozoites, we next performed a thorough bioinformatic search for the presence of corresponding enzymes in the *E. falciformis* genome (www.eupathdb.org). The query sequences included enzymes that have been characterized from various eukaryotic and prokaryotic organisms. We could identify a total of 20 enzymes potentially involved in lipid synthesis of *E. falciformis* (Table 2 and Appendix 1). Some enzymes, PtdGro-phosphate phosphatase (PGPP), ethanolamine phosphotransferase (EPT), PtdEtn methyltransferase (PEMT) and IPC synthase could not be found in *E. falciformis* despite their prediction by aforementioned lipidomics analyses. We also compared the repertoire of lipid synthesis genes present in *E. falciformis* with other parasitic protists. Our results showed that three coccidian parasites *E. falciformis*, *T. gondii* and *Neospora caninum* shared similar inventory, which was more intricate than other apicomplexans (*Plasmodium* and *Cryptosporidium* sp.), as well as kinetoplastid parasites (*Trypanosome* and *Leishmania* sp.). For example, *Eimeria*, *Toxoplasma* and *Neospora* all possessed a PtdThr synthase (PTS), absent in other parasites (Table 2). Likewise, many enzymes occur as two or even three distinct isoforms in coccidians, *e.g.*, we detected 3 paralogs for DAG kinase (DGK) and choline/ethanolamine phosphotransferase (CEPT), as well as 2 isoforms of PtdSer decarboxylase (PSD) and each of three enzymes synthesizing PtdOH and CDP-DAG, glycerol-3P acyltransferase (G3PAT), Lyso-PtdOH acyltransferase (LPAAT) and CDS.

We were able to clone and experimentally annotate the full-length sequence of 18 enzymes using the RNA isolated from *Eimeria* sporozoites, which confirmed the transcription of nearly all lipid synthesis genes. Two open reading frames encoding for *Ef*DGK3 and *Ef*CEPT3 could not be amplified from the parasite RNA. Primary structures of the cloned enzymes revealed that all of them harbor an intact catalytic domain with conserved residues required for substrate/cofactor binding (Figure 6, Appendix 2-3). Most proteins except for LPAAT2, DGK1/2, PtdGro-phosphate synthase (PGPS), cardiolipin synthase (CLS), PSD1/2 and *Ef*CEPT3 harbor one or more defined transmembrane regions, signifying their membrane-binding nature. Notably, G3PAT1, DGK2 and PSD2 contain a predicted (secretory) signal peptide at

their N-terminal. Likewise, the N-termini of PGPS, CLS and PSD1 comprise a mitochondrial targeting peptide (Figure 6). Taken together, the results suggest expression of an almost complete network for *de novo* synthesis of major phospholipids with distinct subcellular distribution in sporozoites.

Table 2. EuPathDB accession numbers of the enzymes involved in phospholipid biogenesis of selected protozoan parasites.

	Eimeria falciformis (Bayer Haberkorn 1970)	*Toxoplasma gondii* (GT1)	*Neospora caninum* (Liverpool)	*Plasmodium berghei* (ANKA)	*Plasmodium falciparum* (3D7)	*Cryptosporidium parvum* (Iowa II)	*Trypanosoma brucei* (TREU927)	*Trypanosoma cruzi* (CL Brener Non-Esmeraldo-like)	*Leishmania major* (Friedlin)
Glycerol 3-Phosphate Acyltransferase (G3PAT)	EfaB_PLUS_45924.g2677 EfaB_MINUS_3469.g362	TGGT1_256980 TGGT1_270910	NCLIV_029980 NCLIV_035870	PBANKA_1428500 PBANKA_1416700	PF3D7_1212500 PF3D7_1318200	cgd6_1270	Tb927.10.3100	TcCLB.510943.150	LmjF.03.0080
Lysophosphatidic Acid Acyltransferase (LPAAT)	EfaB_MINUS_17400.g1567 EfaB_MINUS_12065.g1058	TGGT1_240860 TGGT1_297640	NCLIV_017010 NCLIV_006550	PBANKA_1308200	PF3D7_1444300	cgd8_1400	Tb927.11.15150	TcCLB.510723.29	LmjF.32.1960
CDP-Diacylglycerol Synthase (CDS)	EfaB_PLUS_1048.g115 EfaB_PLUS_36188.g2485	TGGT1_281980 TGGT1_263785	NCLIV_023660 NCLIV_024160	PBANKA_1032600	PF3D7_1409900	cgd7_450	Tb927.7.220	TcCLB.511237.40 TcCLB.508707.140	LmjF.26.1620 LmjF.32.2870
Phosphatidic Acid Phosphatase (PAP)	EfaB_MINUS_22960.g1971	TGGT1_247360	NCLIV_063870	-	PF3D7_0805600	cgd8_5200	Tb927.8.480 Tb927.10.13930 Tb927.10.13400	TcCLB.503809.110 TcCLB.511277.370 TcCLB.511277.359	LmjF.18.0440
Diacylglycerol Kinase (DGK)	EfaB_MINUS_7048.g670 EfaB_MINUS_36188.g2575 EfaB_MINUS_11882.g1052	TGGT1_202460 TGGT1_259830 TGGT1_239250	NCLIV_022470 NCLIV_027060 NCLIV_015910	PBANKA_1334600 PBANKA_0831200	PF3D7_1471400 PF3D7_0930500	cgd4_4340 cgd8_2390 cgd3_2630	Tb927.8.5140	TcCLB.506575.60 TcCLB.510329.290	LmjF.16.1290 LmjF.35.5370
Phosphatidylglycerophosphate Synthase (PGPS)	EfaB_MINUS_17907.g1626	TGGT1_246530	NCLIV_063320	PBANKA_0710600	PF3D7_0820200	-	Tb927.8.1720	TcCLB.505071.100	LmjF.07.0200
Phosphatidylglycerophosphate Phosphatase (PGPP)	-	-	-	-	-	-	-	-	-
Cardiolipin Synthase (CLS)	EfaB_PLUS_56725.g2797	TGGT1_309940	NCLIV_054060	PBANKA_0108000	PF3D7_0609400	cgd3_2940	Tb927.4.2560	TcCLB.506559.40	LmjF.34.2110
Phosphatidylinositol Synthase (PIS)	EfaB_PLUS_6035.g572	TGGT1_207710	NCLIV_002660	PBANKA_1414100	PF3D7_1315600	cgd8_560	Tb927.9.1610	TcCLB.503925.80	LmjF.26.2480
Choline/Ethanolamine Phosphotransferase (CEPT)	EfaB_MINUS_56725.g2919 EfaB_MINUS_15745.g1427 EfaB_MINUS_25458.g2174	TGGT1_257510 TGGT1_261760 TGGT1_276190	NCLIV_029590 NCLIV_025720 NCLIV_006960	PBANKA_1127000	PF3D7_0628300	cgd4_2790 cgd4_390	Tb927.10.8900	TcCLB.509791.150	LmjF.36.5900
Ethanolamine Phosphotransferase (EPT)	-	-	-	-	-	-	Tb927.10.13290	TcCLB.503681.20	LmjF.18.0810
Phosphatidylethanolamine N-Methyltransferase (PEMT)	-	-	-	-	-	-	-	-	LmjF.31.3120 LmjF.31.2290
Phosphatidylserine Decarboxylase (PSD)	EfaB_MINUS_22450.g1948 EfaB_MINUS_1072.g165	TGGT1_225550 TGGT1_269920	NCLIV_047160 NCLIV_036570	PBANKA_0828700	PF3D7_0927900	cgd3_2100	Tb927.9.10080	TcCLB.407335.9	LmjF.35.4590
Phosphatidylserine Synthase (PSS)	EfaB_PLUS_1974.g212	TGGT1_261480	NCLIV_026010	PBANKA_1142700	PF3D7_1366800	cgd1_1110	Tb927.7.3760	TcCLB.509937.30	LmjF.14.1200
Phosphatidylthreonine Synthase (PTS)	EfaB_MINUS_800.g81	TGGT1_273540	NCLIV_034110	-	-	-	-	-	-

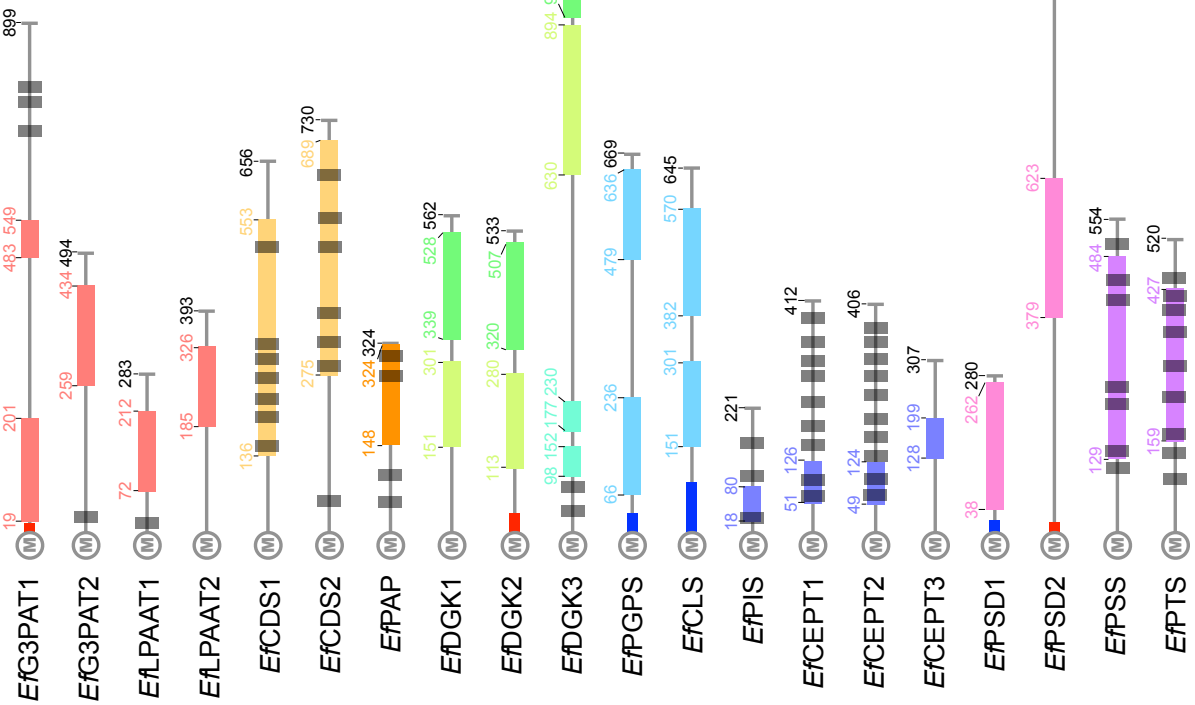


Figure 6. Schematic illustration showing the primary structures of putative enzymes in the phospholipid synthesis pathways of *E. falciformis*. Predicted functional domains, transmembrane regions, as well as signal and mitochondrial targeting peptides are shown with boxes in different colors as indicated in the figure. The numbers represent the positions of the functional domains and the lengths of the proteins. The length of domains and enzymes are all shown to a proper scale. Structure drawing for the enzymes in *T. gondii* can be found in Appendix 2. For phospholipid synthesis pathways and related enzymes in protozoan parasites, see Figure 3 and Table 2. *Abbreviations*: CDS, cytidine diphosphate-diacylglycerol synthase; CEPT, choline/ethanolamine phosphotransferase; CLS, cardiolipin synthase; DGK, diacylglycerol kinase; G3PAT, glycerol 3-phosphate acyltransferase; LPAAT, lysophosphatidic acid acyltransferase; PAP, phosphatidic acid phosphatase; PGPS, phosphatidylglycerol phosphate synthase; PIS, phosphatidylinositol synthase; PSD, phosphatidylserine decarboxylase; PSS, phosphatidylserine synthase; PTS, phosphatidylthreonine synthase.

3.1.3. Lipid synthesis in *E. falciformis* is a phylogenetic mosaic of divergent pathways

We performed the phylogenetic clustering of selected enzymes involved in both eukaryotic and prokaryotic lipid biogenesis pathways to determine their evolutionary origins. G3PAT, LPAAT, CDS, PGPS/CLS, PSD and PtdSer synthase (PSS)/PTS were subjected to cladogram analysis with respective orthologs from all major domains of the tree of life (Figure 7, see Appendix 4 for phylogeny trees of other enzymes). *Ef*G3PAT1 and *Ef*LPAAT1 segregated with their corresponding homologs from other protozoan parasites, whereas *Ef*G3PAT2 and *Ef*LPAAT2 clustered with homologs from red and green algae and plants (Figure 7A-B). Similar phylogenetical pattern also applied to *Ef*CDS1 and *Ef*CDS2, of which the latter grouped with CDSs from not only algae and plants but also cyanobacteria (Figure 7C). In the PtdGro and cardiolipin synthesis pathway, two enzymes (*Ef*PGPS and *Ef*CLS) were identified in *E. falciformis*, both of which encompassed the classic duplicated phospholipase D-like domains (see Figure 6). They and their protozoan homologs clustered with PGPSs from animals and fungi, as well as prokaryotic CLSs, forming the phospholipase-D-type clade (Figure 7D). The rest of PGPS and CLS sequences belonged to the CDP-alcohol-phosphotransferase-type clade. The two PSDs from *E. falciformis* (*Ef*PSD1 and *Ef*PSD2) grouped in different clades. *Ef*PSD1 orthologs appear conserved across phyla in the eukaryotic-type-I clade, while *Ef*PSD2-type proteins could only be identified in related coccidian parasites (Figure 7E). Phylogenetic analysis of *Ef*PSS and *Ef*PTS demonstrated that they both belong to the base-exchange-type enzymes unlike CDP-DAG-dependent bacterial and fungal counterparts. Again, *Ef*PSS orthologs are present across the domains of life, whereas *Ef*PTS-type enzymes were found only in selected coccidians (Figure 7F). These data together indicate a surprising occurrence of fairly divergent enzyme isoforms in *Eimeria*, which have likely been acquired and repurposed to serve the specialized parasitic lifestyle.

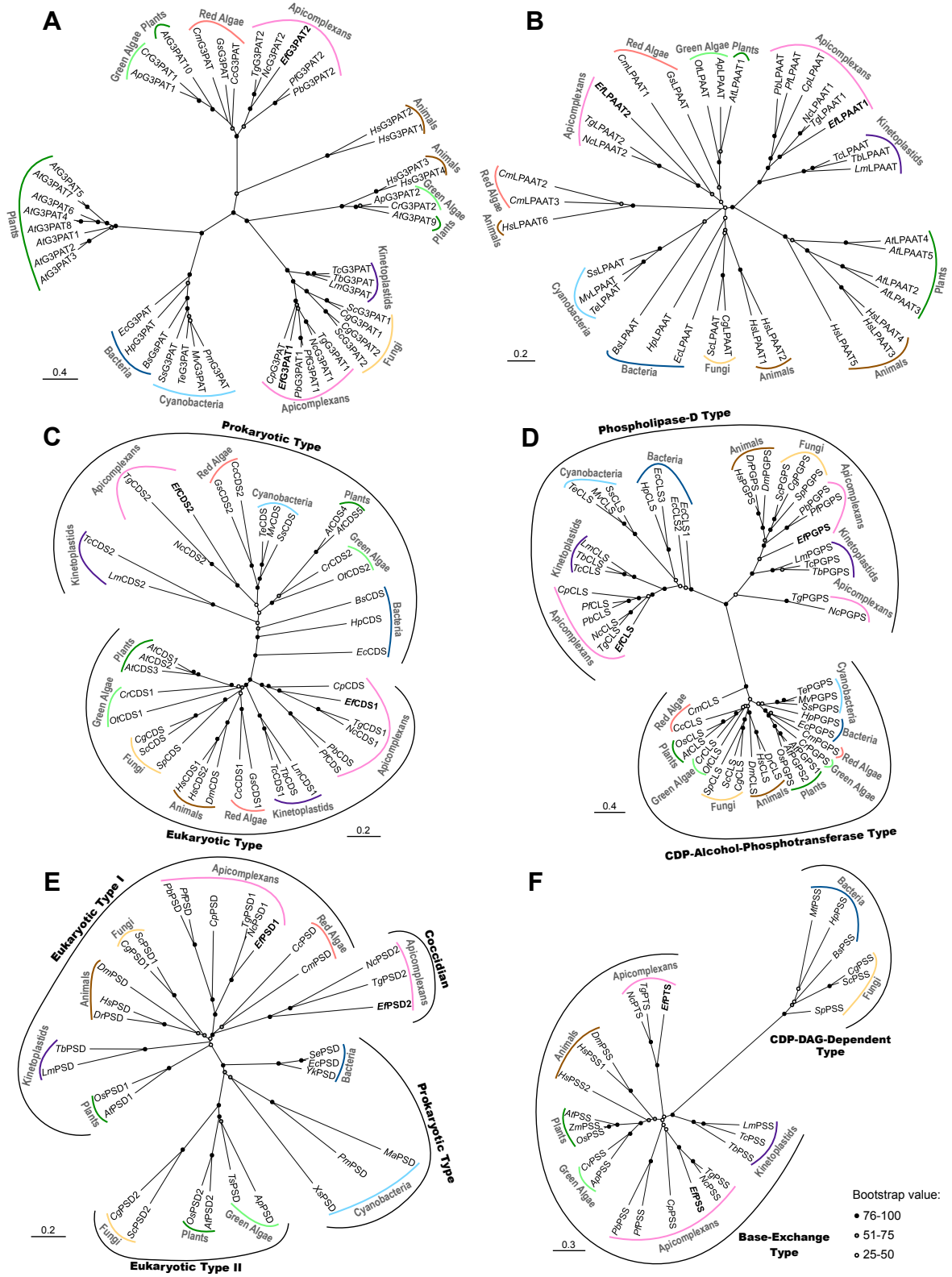


Figure 7. The phospholipid biogenesis in *E. falciformis* involves enzymes of various origins. Phylogenetic trees show the evolutionary relationships of G3PAT (**A**), LPAAT (**B**), CDS (**C**), PGPS/CLS (**D**), PSD (**E**) and PSS/PTS (**F**) enzymes in *E. falciformis* and various organisms representing major tree of life. Branch support was estimated by 100 bootstrap replicates. See Appendix 4 for phylogeny trees of other enzymes. Sequence information including accession numbers and full organism names are shown in Appendix 5. *Abbreviations*: CDS, cytidine diphosphate-diacylglycerol synthase; CLS, cardiolipin synthase; G3PAT, glycerol 3-phosphate acyltransferase; LPAAT, lysophosphatidic acid acyltransferase; PGPS, phosphatidylglycerol phosphate synthase; PSD, phosphatidylserine decarboxylase; PSS, phosphatidylserine synthase; PTS, phosphatidylthreonine synthase.

3.1.4. Enzymes of *Eimeria* lipid synthesis show compartmentalized distribution in *T. gondii*

Next, we determined subcellular distributions of aforementioned enzymes from *E. falciformis*. To this end, we performed ectopic overexpression of epitope-tagged proteins in tachyzoites of *T. gondii* because *in vitro* culture of *E. falciformis* and methods for its genetic manipulation have not been established yet. Transgenic tachyzoites expressing *Eimeria* enzymes tagged with a C-terminal HA epitope were co-localized with pertinent organelle markers by indirect immunofluorescence assay (Figure 8A).

*Ef*G3PAT1-HA and *Ef*CDS1-HA were targeted to the ER (Figure 8B and 8F), whereas *Ef*G3PAT2 and *Ef*CDS2 were found in the apicoplast (Figure 8C and 8G), which resonates with the phylogenetic origin of both enzymes and the evolutionary trace of this organelle. *Ef*LPAAT1-HA was expressed primarily in the Golgi bodies with a week staining in the ER (Figure 8D). Surprisingly, *Ef*LPAAT2 was localized in the ER despite its algal origin (Figure 8E), indicating an incomplete apicoplast pathway and potential transport of Lyso-PtdOH and PtdOH between ER/Golgi and apicoplast. After CDP-DAG is synthesized in the apicoplast and ER, it serves as the precursor for the biogenesis of PtdGro, cardiolipin and PtdIns (Figure 3). The two enzymes in the PtdGro and cardiolipin pathway, *Ef*PGPS and *Ef*CLS, were expressed in the mitochondrion (Figure 8H-I), whilst the PtdIns synthase (*Ef*PIS) was localized in the Golgi bodies (Figure 8J). These data demonstrate that transfer of CDP-DAG from the site of its synthesis to the mitochondrion and Golgi bodies would be required for the downstream lipid synthesis.

In eukaryotes, PtdOH is not only used for CDP-DAG production, but also for the synthesis of DAG, which is regulated by two reactions catalyzed by PtdOH phosphatase (PAP) and DGK (Figure 3). *Ef*PAP-HA displayed punctate intracellular distribution (Figure 8K), while *Ef*DGK1-HA and *Ef*DGK2-HA localized in the parasite periphery and DG/PV, respectively (Figure 8L-M). DAG serves as a co-substrate for CEPT to synthesize PtdCho and PtdEtn *via* the Kennedy pathways (Figure 3). Unexpectedly, both *Ef*CEPT1 and *Ef*CEPT2 were expressed in the Golgi bodies (Figure 8N-O). Alternatively, PtdEtn can be synthesized by decarboxylation of PtdSer under the action of PSD enzymes (Figure 3). *Ef*PSD1 and *Ef*PSD2 were targeted to

the mitochondrion and DG/PV, respectively (Figure 8P-Q). Consistent with localization results, all mitochondrion proteins (*Ef*PGPS, *Ef*CLS and *Ef*PSD1) and DG/PV proteins (*Ef*DGK2 and *Ef*PSD2) harbored corresponding targeting motifs (mitochondrial or secretory; Figure 6). PtdEtn and/or PtdCho are used to produce PtdSer and PtdThr *via* base-exchange reactions catalyzed by PSS and PTS, respectively (Figure 3). Both enzymes were found in the parasite ER with *Ef*PSS showing a strong Golgi signal (Figure 8R-S). Taken together, our localization studies revealed an assorted subcellular distribution of *Eimeria* enzymes in tachyzoites of *T. gondii*.

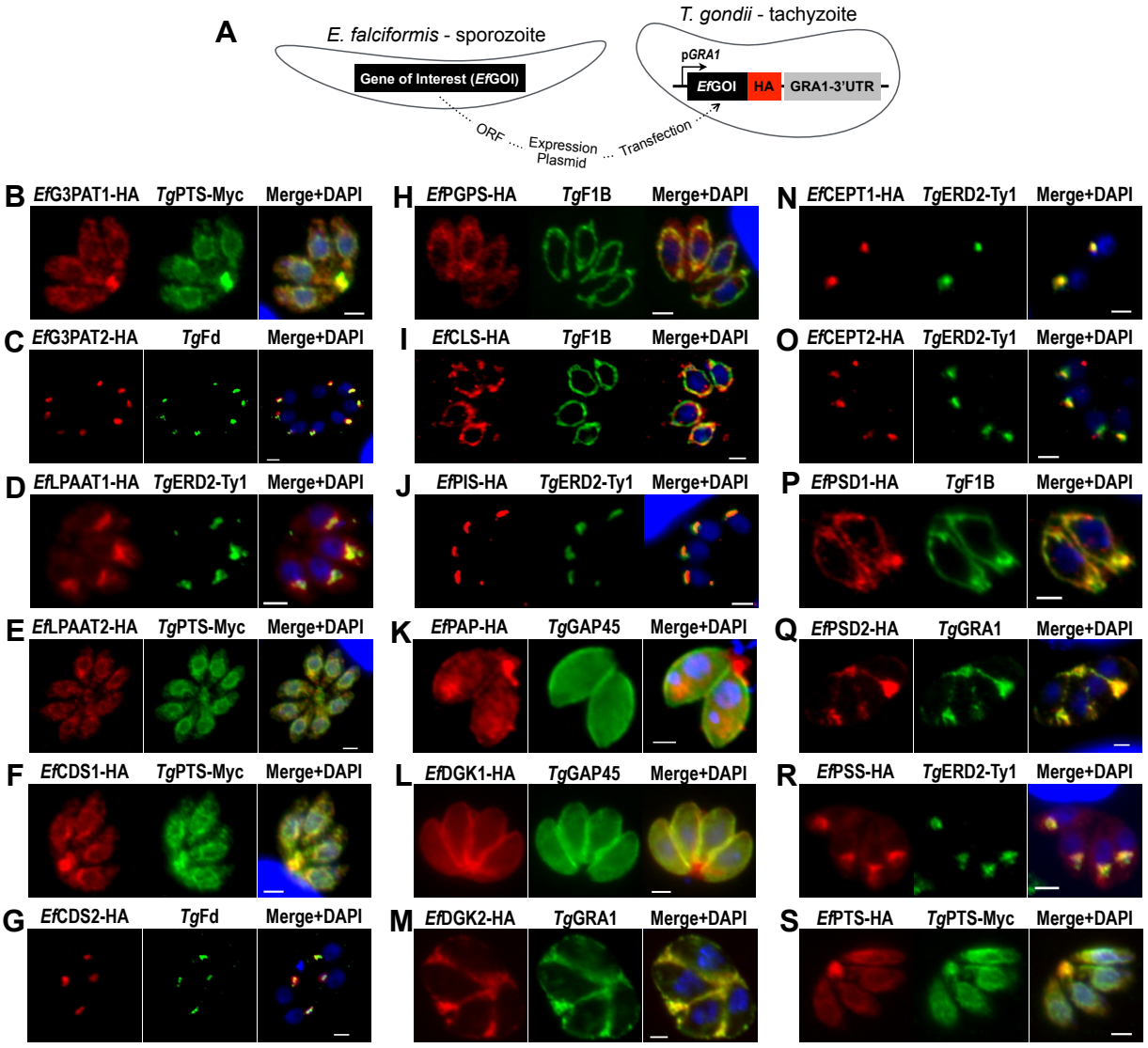


Figure 8. Syntheses of phospholipids and their precursors are highly compartmentalized in *E. falciformis*. (**A**) Selected *E. falciformis* enzymes were ectopically expressed in *T. gondii* tachyzoites under the control of *Tg*GRA1 promoter and 3'UTR. Immunofluorescence was performed at 24 h post infection to detect *Ef*G3PAT1-HA (**B**), *Ef*G3PAT2-HA (**C**), *Ef*LPAAT1-HA (**D**), *Ef*LPAAT2-HA (**E**), *Ef*CDS1-HA (**F**), *Ef*CDS2-HA (**G**), *Ef*PGPS-HA (**H**), *Ef*CLS-HA (**I**), *Ef*PIS-HA (**J**), *Ef*PAP-HA (**K**), *Ef*DGK1-HA (**L**), *Ef*DGK2-HA (**M**), *Ef*CEPT1-HA (**N**), *Ef*CEPT2-HA (**O**), *Ef*PSD1-HA (**P**), *Ef*PSD2-HA (**Q**), *Ef*PSS-HA (**R**) and *Ef*PTS-HA (**S**) using anti-HA and Alexa594 antibodies. The subcellular localizations of each enzyme were confirmed by co-localization with corresponding organelle markers including *Tg*PTS-Myc for ER, *Tg*Fd for apicoplast, *Tg*ERD2-Ty1 for Golgi body, *Tg*F1B for mitochondrion, *Tg*GAP45 for parasite periphery, as well as *Tg*GRA1 for DG and PV. Scale bars: 2 µm. *Abbreviations*: CDS, cytidine diphosphate-diacylglycerol synthase; CEPT, choline/ethanolamine phosphotransferase; CLS, cardiolipin synthase; DGK, diacylglycerol kinase; ERD2, endoplasmic reticulum deficient mutant 2; F1B, ATPase subunit F1-B; Fd, ferredoxin; G3PAT, glycerol 3-phosphate acyltransferase; GAP45, glideosome-associated protein 45; GRA1, dense granule protein 1; LPAAT, lysophosphatidic acid acyltransferase; PAP, phosphatidic acid phosphatase; PGPS, phosphatidylglycerol phosphate synthase; PIS, phosphatidylinositol synthase; PSD, phosphatidylserine decarboxylase; PSS, phosphatidylserine synthase; PTS, phosphatidylthreonine synthase.

3.1.5. Trans-species expression of *Ef*PTS rescues the lytic cycle of the Δ*tgpts* mutant

Next, we tested the functionality of *Ef*PTS, a novel coccidian-specific enzyme, in a mutant of *T. gondii* lacking PTS expression. Our recent study has shown that the disruption of the PTS in tachyzoites (Figure 9A - Step 1 and Appendix 3F) ablates the synthesis of PtdThr, which in turn compromises the parasite motility, egress and invasion, leading to impairment of the lytic cycle [50]. The Δ*tgpts* mutant therefore offered a tool to test the catalytic function and physiological role of *Ef*PTS. The enzyme was expressed under the control of p*GRA1* promoter at the *TgUPRT* locus in the Δ*tgpts* mutant (Figure 9A - Step 2). The eventual complemented strain (Δ*tgpts*/*Ef*PTS) was subjected to phenotypic assays to elucidate the physiological impact of *Ef*PTS expression. The Δ*tgpts* mutant formed significantly smaller (-73%) and less (-69%) plaques compared to the parental strain in the plaque assay (Figure 9B-D), confirming the earlier work [50]. Quite notably, *Ef*PTS completely restored the lytic cycle of the PTS mutant. In-depth phenotyping of the Δ*tgpts* strain revealed an evident impairment in its invasion, egress and motility, but not in replication (Figure 9E-J), as reported preciously [50]. The parasite invasion, egress as well as gliding motility were reinstated in the complemented strain. Remarkably, ectopic expression of *Ef*PTS also conferred an increased proliferation to the mutant (Figure 9F). These assays together confirm that *Ef*PTS can compensate for the loss of its counterpart enzyme in *T. gondii* tachyzoites.

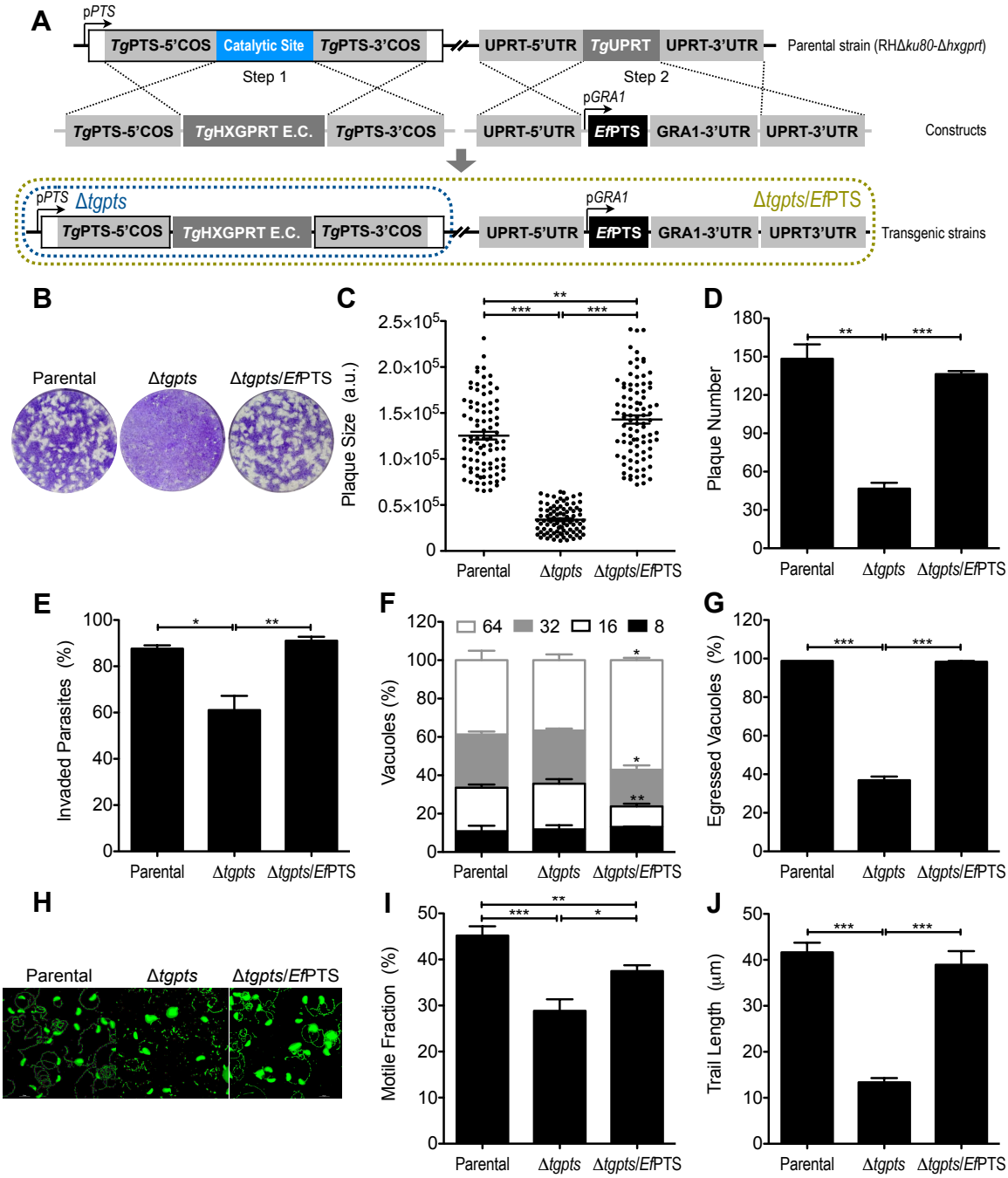


Figure 9. *Ef*PTS can complement the defects of invasion, egress and motility in a PTS-knockout mutant of *T. gondii*. The Δ*tgpts* mutant was created by Ruben D. Arroyo-Olarte *et al* and found defective in invasion, egress and motility, but not in replication [50]. (**A**) Scheme shows targeted disruption of the catalytic site of the *Tg*PTS gene (Step 1) and insertion of *Ef*PTS (Step 2). To achieve the Δ*tgpts*/*Ef*PTS strain, *Ef*PTS was integrated at the *TgUPRT* locus under the control of p*GRA1* promoter in the Δ*tgpts* mutant *via* double homologous recombination. (**B**-**D**) Plaque assays recapitulate successive lytic cycles of tachyzoites in host cells and display the *in vitro* growth fitness of the indicated parasite strains. *Ef*PTS complements the Δ*tgpts* mutant in both plaque size (C) and number (D) from 3 independent experiments. The Δ*tgpts*/*Ef*PTS strain also shows similar rate with the parental strain in invasion assays (**E**), egress assays (**G**) and motility assays (**H**-**J**), and even a higher rate in the replication assay (**F**). For *panel C*, 90 plaques of each strain from 3 assays were measured for area using the ImageJ software. For *panel E*, the invasion rates of 1500-2500 parasites of each strain from 3 assays were presented. For *panel F* and *G*, a total of 200–350 vacuoles for each strain from 3 assays were analyzed for the percentages of either vacuoles containing different numbers of parasites (F) or natural egress (G). For *panel I* and *J*, 30-60 parasites of each strain were scored from 3-6 assays in total for their motile fractions (I) and trail lengths (J). In all assays, values are means with SEM (*p < 0.05, **p < 0.01, ***p < 0.001).

3.1.6. *Ef*PTS can restore the loss of tachyzoite-specific PtdThr species in the Δ*tgpts* mutant

As mentioned above, the composition of PtdThr species were notably different between *E. falciformis* sporozoites and *T. gondii* tachyzoites (Figure 5C). The most abundant PtdThr species in *E. falciformis* sporozoites was C36:1 (41%), followed by C36:2 (26%), C38:4 (10%), C34:1 (7%), C40:6 (6%) and C38:2 (6%) (Figure 5C and 10A). In *T. gondii* tachyzoites, however, all detected PtdThr species contained polyunsaturated fatty acid chains, including C40:5 (62%), 38:4 (12%), 36:4 (11%), 40:6 (10%) and 38:5 (4%) (Figure 5C and 10B). The MS/MS analysis identified 18:0/18:1 and 20:1/20:4 as the most prevalent PtdThr species in *E. falciformis* sporozoites and in the parental tachyzoites of *T. gondii*, respectively (Figure 10C-D). As expected, lipidomics of the Δ*tgpts* strain confirmed the lack of all PtdThr species. Very surprisingly, complementation of the PTS mutant with *Ef*PTS fully restored PtdThr content; however with the same species as present in the parental tachyzoites (Figure 10E) but not with those present in *Eimeria*. Interestingly, *Ef*PTS does not produce 18:0/18:1 PtdThr in tachyzoites even though the fatty acid is readily available, suggesting a specific need of PtdThr containing polyunsaturated acyl chains (mainly 20:1/20:4) for the lytic cycle of *T. gondii*. These data also demonstrate that *Ef*PTS encodes a functional enzyme with wide specificity for substrates and it is capable of synthesizing different PtdThr species in a new environment.

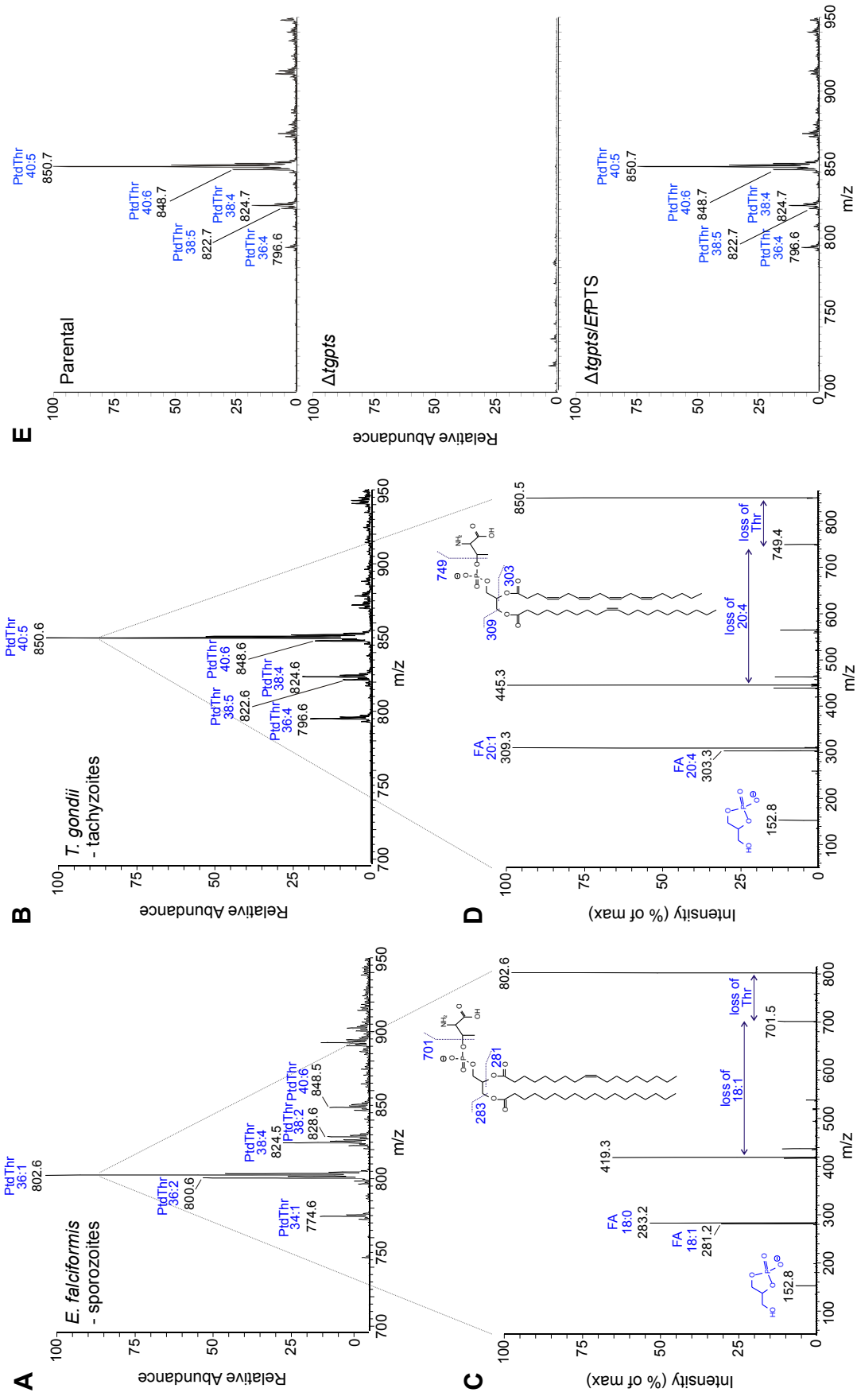


Figure 10. *Ef*PTS rescues the loss of tachyzoite-specific PtdThr species in the Δ*tgpts* mutant. (**A-B**) MS analysis identifies the most abundant species of PtdThr in *E. falciformis* sporozoites is 36:1, followed by 36:2, while the dominant PtdThr species in *T. gondii* tachyzoites is 40:5. Individual PtdThr species was identified by their fragmentation patterns and m/z ratios in the negative ionization mode. (**C-D**) MS/MS study shows the spectrum of the dominant PtdThr peaks (m/z 802.6 for *E. falciformis* and m/z 850.6 for *T. gondii*) from *panel A* and *B*. Acyl chains were identified by their masses as C36:1 (18:0/18:1) and C40:5 (20:1/20:4). (**E**) MS analysis reveals the dominant PtdThr species (40:5) and minor species (40:6, 38:4, 38:5 and 36:4) in *T. gondii* parental strain corresponding to *panel B*, which are all absent in the Δ*tgpts* mutant. The loss of all detectable PtdThr species is restored by expression of *Ef*PTS in the Δ*tgpts*/*Ef*PTS strain.

3.2. Two phylogenetically and compartmentally distinct CDP-diacylglycerol synthases cooperate for lipid biogenesis in *T. gondii*

3.2.1. *T. gondii* harbors two phylogenetically distinct CDS proteins

Next, we used the well-established model organism *T. gondii* to study the phylogenetic and metabolic roles of CDS, the central enzyme in lipid synthesis network, in details. Our bioinformatics searches using *bona fide* CDS protein sequences from yeast and human identified one CDS gene in *Toxoplasma* database (www.ToxoDB.org), termed as *Tg*CDS1 henceforth (ToxoDB, TGGT1_281980; GenBank, KU199242). Similar database mining using the prokaryotic CDS sequences indicated the unexpected existence of a second CDS in the parasite, which was designated as *Tg*CDS2 (ToxoDB, TGGT1_263785; GenBank KU199243). Subsequently, we also found prokaryotic-type CDSs in selected protozoan parasites *E. falciformis*, *N. caninum*, *Trypanosoma cruzi* and *Leishmania major* (Table 2), albeit not in many others (*Plasmodium berghei*, *Plasmodium falciparum*, *Cryptosporidium parvum* and *Trypanosoma brucei*). Phylogenetic clustering of CDS sequences revealed discrete eukaryotic and prokaryotic clades (Figure 11A and 7C). All eukaryotes possessed at least one CDS, which clustered with *Tg*CDS1. In contrast, *Tg*CDS2 segregated with prokaryotic-type CDS sequences from bacteria, plants, algae, and indicated parasites. Notably, *Tg*CDS2 grouped with CDSs from cyanobacteria (*Pm*CDS, *Ss*CDS and *Te*CDS) and red alga (*Cc*CDS2 and *Gs*CDS2), both are considered as ancestors of the apicoplast in apicomplexan parasites (Figure 11A and 7C).

The ORFs of *Tg*CDS1 and *Tg*CDS2 encode for 1068 and 1044 amino acids with several transmembrane regions (Figure 11B). Both proteins contain an archetypal cytidylyltransferase domain encompassing a CDS signature motif (G-X_4-S-X_2-KR-X_4-KD-X_5-PGHGG-X_2-DR-X-D, Figure 11B-C). These features are also shared by homologs from other indicated organisms irrespective of the phylogenetic origins. Sequence comparisons also revealed many signature residues that were differentially conserved in eukaryotic- and prokaryotic-type CDS sequences (see red and blue-shaded residues, Figure 11C). In particular, prokaryotic-type proteins harbor acidic and basic residues (aspartate-lysine, DL) instead of small and aromatic pair of amino acids (glycine-phenylalanine, GF) occurring in eukaryotic-type

orthologs. Likewise, eukaryotic CDSs show a strictly conserved nucleophile-amide motif (cysteine-glutamine, CQ) absent in prokaryotic counterparts.

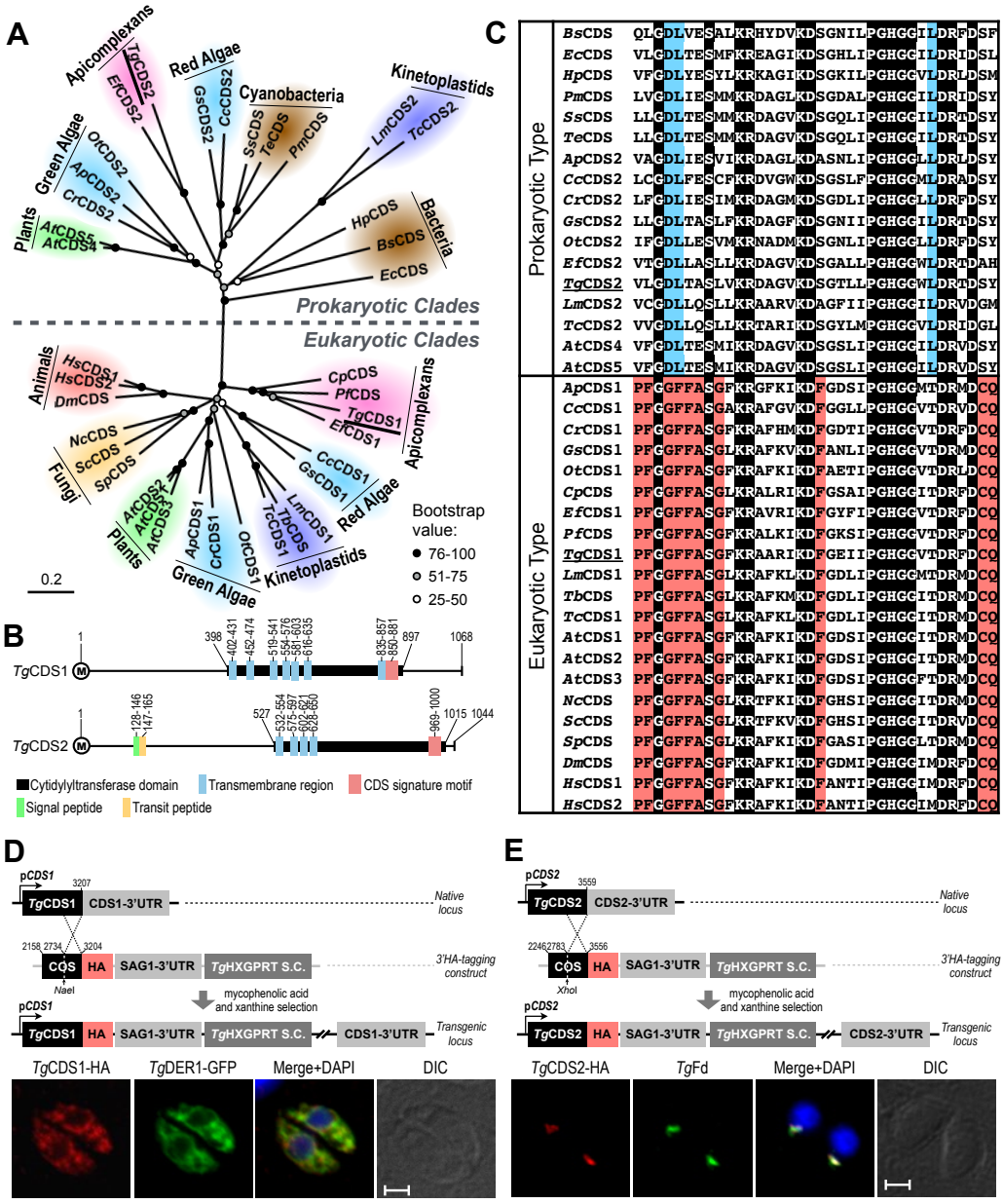


Figure 11. *T. gondii* expresses two distinct CDS enzymes located in the ER and apicoplast. (**A**) Phylogenetic analysis of *Tg*CDS1, *Tg*CDS2 and orthologs from various organisms representing major tree of life. Branch support was estimated by 100 bootstrap replicates. Other relevant information including accession numbers and full organism names for each CDS sequence is shown in Appendix 5. Similar phylogenetic tree is also presented in Figure 7C. (**B**) Schematic drawing of the primary structures of *Tg*CDS1 and *Tg*CDS2. The numbers indicate positions of cytidylyltransferase domains, CDS signature motifs, transmembrane regions, as well as signal and transit peptides. (**C**) Multiple alignments of the signature motifs present in eukaryotic- and prokaryotic-type CDS sequences. The residues identical across all sequences are shaded with black color, and amino acids that are conserved only in eukaryotes or prokaryotes are highlighted in red or blue colors, respectively. (**D**-**E**) Scheme for 3'-insertional tagging of *Tg*CDS1 and *Tg*CDS2 genes with a C-terminal HA tag. Plasmids harboring crossover sequence (COS) of respective genes were linearized with the indicated enzymes and transfected into tachyzoites (RHΔ*ku80*-Δ*hxgprt*), followed by drug selection. Immunofluorescence of stable transgenic tachyzoites expressing *Tg*CDS1-HA (D) and *Tg*CDS2-HA (E) under the control of respective endogenous promoters and *Tg*SAG1-3'UTR was performed using anti-HA and Alexa594 antibodies (24 h post-infection). *Tg*CDS1-HA and *Tg*CDS2-HA proteins were co-localized with *Tg*DER1-GFP signal and anti-*Tg*Fd/Alexa488 antibodies, respectively. Scale bars: 2 μm.

3.2.2. *Tg*CDS1 is expressed in the ER, whereas *Tg*CDS2 in the apicoplast

The parasite database indicated a constitutive expression of *Tg*CDS1 and *Tg*CDS2 transcripts during the lytic cycle (www.ToxoDB.org). To test whether *Tg*CDS1 and *Tg*CDS2 proteins are indeed expressed during the asexual development of tachyzoites, we tagged them with a HA epitope by 3'-insertional tagging of the endogenous loci (Figure 11D-E). The same approach also enabled us to determine subcellular distributions in intracellular parasites by immunofluorescence assays. Consistent with transcript abundance, endogenous expression of *Tg*CDS1 and *Tg*CDS2 was readily detectable in stable transgenic strains. *Tg*CDS1-HA was expressed primarily in the parasite ER, as confirmed by its co-localization with a known organelle marker *Tg*DER1-GFP [82] (Figure 11D). In contrast, *Tg*CDS2-HA co-localized with a *bona fide* marker of apicoplast *Tg*Fd [72] (Figure 11E). Distinct subcellular locations of both CDSs in *T. gondii* are consistent with their respective phylogenetic origins.

The alignment of primary structures revealed prolonged N-terminal extensions in *Tg*CDS1 and *Tg*CDS2 when compared to homologs (Figure 12A). Additionally, a putative bipartite sequence composed of a signal peptide and a transit peptide was identified in the N-terminal extension of *Tg*CDS2, starting from the second methionine instead of the start codon (Figure 11B and Appendix 6). To address the roles of extended N- termini for subcellular targeting, the two mutant isoforms lacking the designated extensions and fused with a C-terminal Myc tag (*Tg*CDS1$^{398\text{-}1068}$-Myc and *Tg*CDS2$^{527\text{-}1044}$-Myc) were ectopically expressed and localized with corresponding full-length proteins. *Tg*CDS1$^{398\text{-}1068}$-Myc was still targeted to the ER, as discerned by co-staining with wild-type *Tg*CDS1 (Figure 12B). In contrast, *Tg*CDS2$^{527\text{-}1044}$-Myc was not localized in the apicoplast anymore and appeared to be cytosolic instead (Figure 12B), suggesting a crucial function of N-terminal peptide for its correct location. We then generated additional mutants of *Tg*CDS2 protein containing internal deletions of either signal (*Tg*CDS2$_{\Delta SP}$-Myc) or transit peptide (*Tg*CDS2$_{\Delta TP}$-Myc) or both (*Tg*CDS2$_{\Delta BS}$-Myc). Localization studies with these mutants revealed a clear role of bipartite sequence for targeting of *Tg*CDS2 to the apicoplast (Fig. 3B).

To examine whether the N-termini were processed during maturation and targeting of the two proteins, we expressed dual-tagged isoforms containing a Myc tag at the N-terminus and a HA epitope at the C-terminus (Myc-*Tg*CDS1-HA or Myc-*Tg*CDS2-HA). In the case of *Tg*CDS1, both Myc and HA epitopes were distributed in the ER (Figure 12C). However, the N-terminal region of *Tg*CDS2 (Myc-tagged) showed an evidently cytosolic signal, while the C-terminal peptide (HA-tagged) was still localized in the apicoplast (Figure 12C), which suggested that the N-terminus of the protein is cleaved off when the rest of the protein is imported and likely integrated into the organelle membrane.

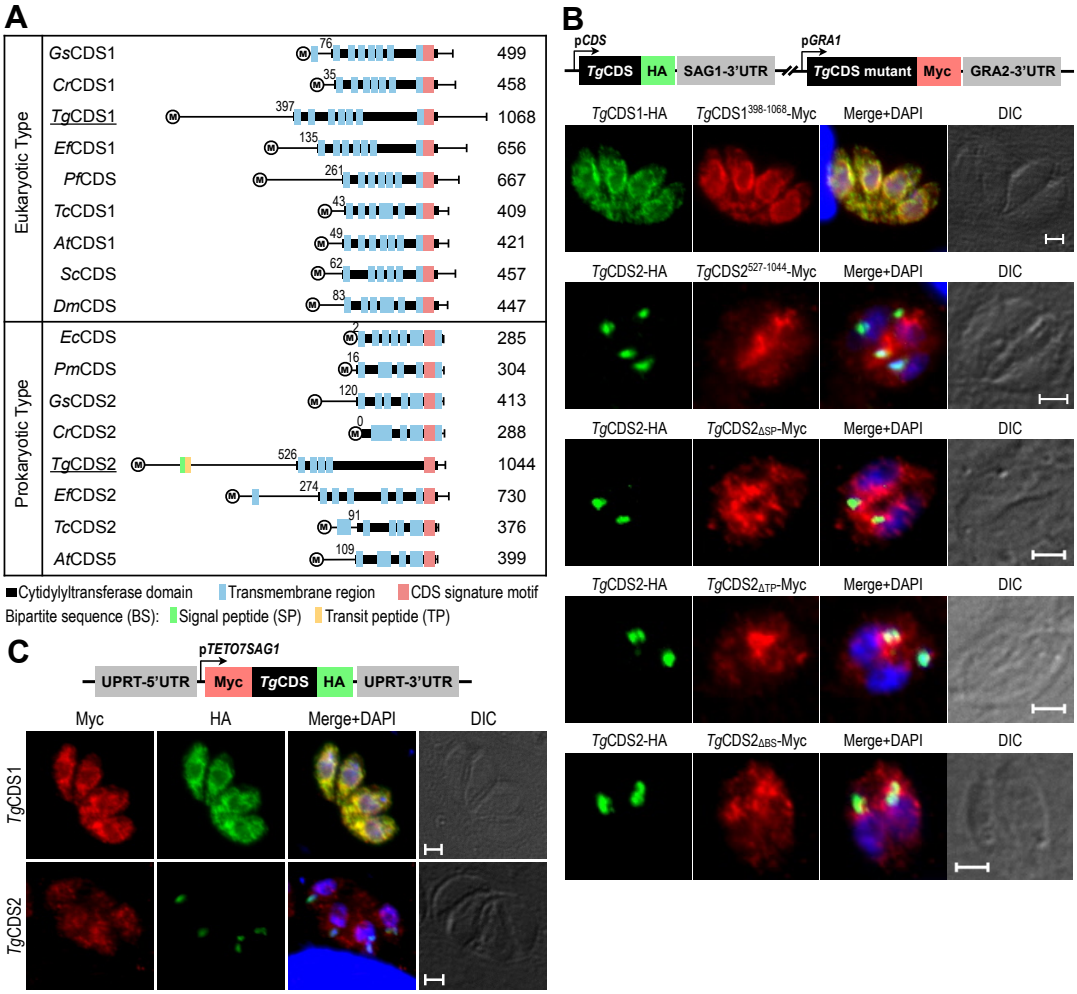


Figure 12. The N-terminal of *Tg*CDS2 but not of *Tg*CDS1 is required for correct localization. (**A**) Comparison of primary structures of CDS orthologs across different organisms. The numbers shown before cytidylyltransferase domains indicate the length of the N-termini. The length of full-length proteins and the position of the bipartite sequence in *Tg*CDS2 are also mentioned. (**B**) Immunofluorescence of tachyzoites co-expressing full-length CDS (*Tg*CDS1-HA or *Tg*CDS2-HA) along with corresponding truncated isoforms lacking the extended N-terminal region (*Tg*CDS1$^{398-1068}$-Myc or *Tg*CDS2$^{527-1044}$-Myc), signal peptide (*Tg*CDS2$_{\Delta SP}$-Myc), transit peptide (*Tg*CDS2$_{\Delta TP}$-Myc) or the entire bipartite sequence (*Tg*CDS2$_{\Delta BS}$-Myc). Transgenic parasites harboring epitope-tagged wild-type proteins (see Figure 11D-E for details) were transfected with plasmids encoding either of the mutated isoforms (regulated by *Tg*GRA1 promoter). (**C**) Immunofluorescence of intracellular parasites expressing dual-tagged (Myc-*Tg*CDS1-HA or Myc-*Tg*CDS2-HA) under the control of p*TETO7SAG1* promoter. Immunostaining in *panel B* and *C* were performed 24 h post-infection using anti-HA/Alexa488 and anti-Myc/Alexa594 antibodies. Scale bars: 2 μm.

3.2.3. CDS enzymes are indispensable for the lytic cycle of tachyzoites

To investigate the physiological importance of *Tg*CDS1 and *Tg*CDS2 in *T. gondii* tachyzoites, we first attempted to create mutants lacking either of the two genes. Our multiple endeavors to delete the *TgCDS1* and *TgCDS2* loci were futile, indicating essential nature of the two proteins in parasites. We therefore generated a conditional mutant of *Tg*CDS1 using the tetracycline repressor-based system, as described elsewhere [38]. To achieve the mutant, *Tg*CDS1-HA was first expressed under the control of a tetracycline-regulated promoter (p*TETO7SAG1*) at *TgUPRT* locus in the RHΔ*ku80*-TaTi strain (Figure 13A, Step 1 - making of a merodiploid strain). We then replaced the native *TgCDS1* locus by the DHFR-TS selection cassette *via* double homologous recombination (Figure 13A, Step 2 - making of Δ*tgcds1*$_r$ mutant). Genetic deletion of the CDS1 gene was confirmed by recombination-specific PCR screening, which showed the occurrence of 5' and 3' homologous recombination events in the mutant but not in the parental strain (Figure 13B). RT-PCR validated a conditional repression of *Tg*CDS1 transcript by ATc in the Δ*tgcds1*$_r$ strain (Figure 13C). *Tg*CDS1 mRNA was induced 2x under the control of p*TETO7SAG1* promoter, which could be down-regulated by about 14x in ATc-treated cultures. As shown by immunofluorescence and immunoblot analyses (Figure 13D-E), exposure to ATc also repressed the expression of *Tg*CDS1-HA protein, which was undetectable after 4 days of cultures with the drug. Unfortunately, measurements of the CDS activity in intact parasites and cell extract have proven extremely challenging, which prevented us from establishing a correlation between the protein level and the enzymatic activity.

Even though we were unsuccessful in generating a *Tg*CDS2 knockout mutant using the parental strain, we were able to delete the *TgCDS2* locus in the Δ*tgcds1*$_r$ mutant, which was probably due to overexpression of *Tg*CDS1 in the latter strain (Figure 13C). To make a double mutant, a plasmid containing the 5' and 3'UTRs of *Tg*CDS2 flanking the CAT selection marker was transfected in the Δ*tgcds1*$_r$ strain (Figure 13A, Step 3 - making of Δ*tgcds1*$_r$/Δ*tgcds2* mutant). The events of double homologous recombination and integration of the selection marker at the *TgCDS2* locus in the double mutant was verified by crossover-specific PCR (Figure 13B). RT-PCR corroborated the absence of *TgCDS2* mRNA in the Δ*tgcds1*$_r$/Δ*tgcds2* strain (Figure 13C). It was not feasible to test the protein level by immunofluorescence or

immunoblot assays due to unavailability of antibody recognizing *Tg*CDS2. With respect to the transcriptional and translational regulation of *Tg*CDS1, the Δ*tgcds1*$_r$/Δ*tgcds2* mutant behaved akin to the Δ*tgcds1*$_r$ strain (Figure 13C-E). For example, the Δ*tgcds1*$_r$/Δ*tgcds2* mutant also showed 2-fold elevation of *Tg*CDS1 transcript in the *on state* (-ATc), which could be repressed by 14-fold in the *off state* (+ATc). Similarly, *Tg*CDS1 protein was expressed at comparable levels in both mutants (*on state*) and a chemical regulation was achievable within 96 hours.

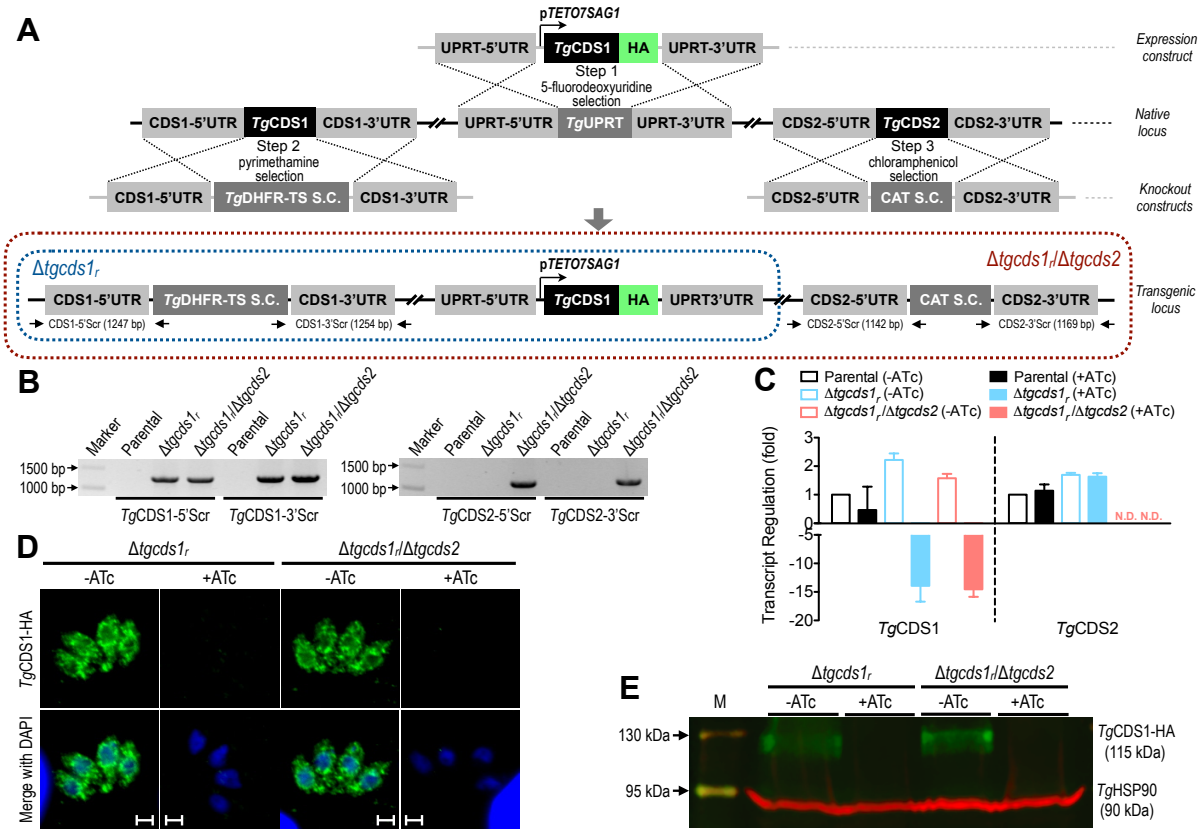


Figure 13. Conditional mutagenesis of *Tg*CDS1 and deletion of *Tg*CDS2 in *T. gondii*. (**A**) Scheme for creating the Δ*tgcds1*$_r$ and Δ*tgcds1*$_r$/Δ*tgcds2* mutants. Step 1 and 2 depict making of the Δ*tgcds1*$_r$ mutant, which involved integrating a tetracycline-regulatable copy of *Tg*CDS1 (*Tg*CDS1-HA) at the *TgUPRT* locus in the Δ*ku80*-TaTi strain (Step 1) followed by deletion of the *Tg*CDS1 gene by the *Tg*DHFR-TS selection cassette (S.C.) *via* double homologous recombination (Step 2). Subsequently, to generate the Δ*tgcds1*$_r$/Δ*tgcds2* strain (Step 3), the *Tg*CDS2 gene was deleted in the Δ*tgcds1*$_r$ mutant by the CAT selection marker. Primers used to screen for 5' and 3' recombination at the *TgCDS1* and *TgCDS2* loci are marked as black arrows. (**B**) Genomic PCR of the Δ*tgcds1*$_r$ and Δ*tgcds1*$_r$/Δ*tgcds2* strains confirming the events of 5' and 3' crossovers. Genomic DNA of the parental strain was included alongside (negative control). (**C**) Quantitative PCR of *Tg*CDS1 and *Tg*CDS2 transcripts in Δ*tgcds1*$_r$ and Δ*tgcds1*$_r$/Δ*tgcds2* mutants. Note that *Tg*CDS1 was induced 2-fold under the control of p*TETO7SAG1* promoter in the *on state* (-ATc) when compared to the parental strain. It was repressed 14-fold in the *off state* (+ATc). *Tg*CDS2 transcript was not detectable (N.D.) in the Δ*tgcds1*$_r$/Δ*tgcds2* strain. The average values with SEM from three independent assays are shown. (**D**-**E**) Immunofluorescence and immunoblot analyses of the two mutants showing a conditional regulation of *Tg*CDS1-HA by ATc. Staining was performed using anti-HA and anti-*Tg*HSP90 antibodies (loading control). Scale bars: 2 µm.

3.2.4. Conditional knockdown of *Tg*CDS1 impairs biogenesis of PtdIns and PtdSer

To discern the functional importance of *Tg*CDS1 and *Tg*CDS2 for phospholipid synthesis, we performed lipidomics analysis. Lipids were isolated from tachyzoites of the parental, Δ*tgcds1$_r$* and Δ*tgcds1$_r$*/Δ*tgcds2* strains cultured in the absence or presence of ATc and subjected to HPLC and MS analysis. As anticipated, total phospholipids in the parental strain were not perturbed by treatment with ATc, whereas we found a modest increase in the Δ*tgcds1$_r$* mutant during *on state*, which was further increased in *off state* (Appendix 7A). In-depth analysis of individual lipids revealed a notable elevation of all major phospholipids, which were probably due to ectopic overexpression of *Tg*CDS1 (Figure 14A and 14C, Appendix 7B-D). More importantly, a knockdown of *Tg*CDS1 in the Δ*tgcds1$_r$* strain led to a significant and rather selective reduction in the amounts of PtdIns and PtdSer (Figure 14A and 14C). Other phospholipids were either unaffected (PtdEtn and PtdThr, Appendix 7C-D) or even elevated (PtdCho in Appendix 7B and PtdGro in Figure 14E). Such a modest rise in PtdCho (accounting for 70-80% of phospholipids [41,45]) was sufficient to induce the level of total lipids during the *off state* despite a prominent decline in PtdIns and PtdSer (Appendix 7A), which together accounted for <10% of total phospholipids.

Next, we examined the major species of PtdIns and PtdSer (Figure 14B and 14D). PtdIns species were comprised of primarily short to medium saturated/unsaturated fatty acids (30-36 carbons). In particular, the amount of the most abundant species 34:1 and 34:2 changed dramatically in the Δ*tgcds1$_r$* mutant in an ATc-regulated manner when compared to the parental strain. Other key species of PtdIns that were declined upon repression of *Tg*CDS1 included 30:0, 32:1 and 36:2. One particular species 36:4 (C16:0/20:4, confirmed by MS/MS) of PtdIns showed a surprisingly opposite trend in the mutant. Interestingly, the most abundant PtdIns species in HFFs is PtdIns 38:4 (C18:0/20:4, confirmed by MS/MS), which is negligible in the parasite extract, leads to a suspicion that tachyzoites might salvage this species containing C20:4 acyl chain from host cells, and then remodel to its own benefit. The most abundant PtdSer species contained mono and polyunsaturated acyl chains with 34, 36 and 42 carbon atoms. All of them were induced noticeably during *on state* of the Δ*tgcds1$_r$* strain, and reduced following a down-regulation of *Tg*CDS1 (*off state*). The

data together suggest a requirement of *Tg*CDS1 to produce PtdIns and PtdSer by downstream CDP-DAG-dependent enzymes.

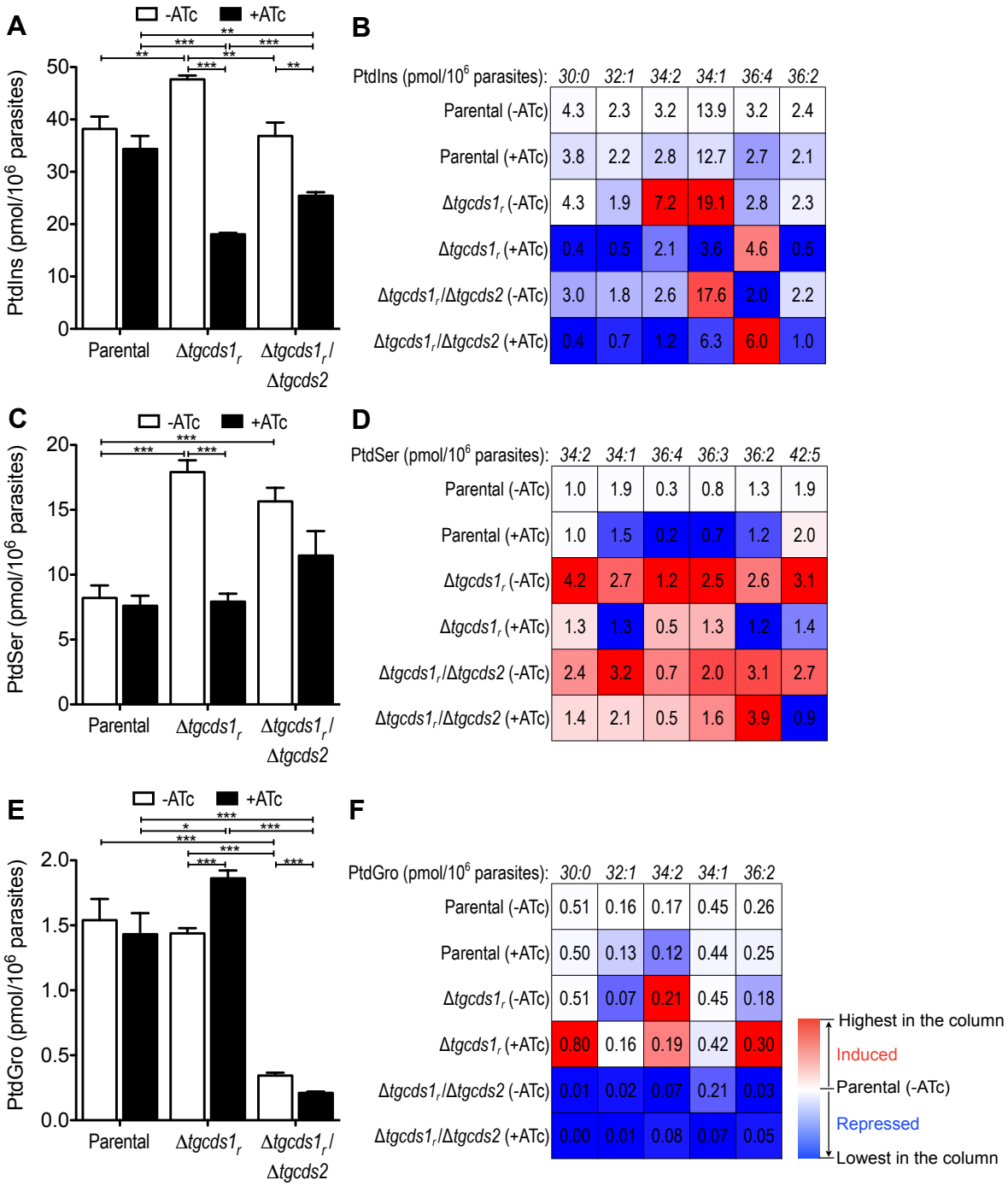


Figure 14. Conditional knockdown of *Tg*CDS1 results in a reduction of PtdIns and PtdSer, whereas deletion of *Tg*CDS2 impairs PtdGro biogenesis. Total lipids were extracted from tachyzoites of the indicated strains and subjected to HPLC and MS analysis. Amounts of PtdIns (**A**), PtdSer (**C**), PtdGro (**E**) and corresponding major lipid species (**B**, **D**, **F**) in the presence or absence of ATc are depicted in heat-maps. Color-coded gradient in each column is meant to compare the relative abundance of the given lipid species in the mutants with respect to the *on-state* parental strain. Actual amounts of lipid species (pmol/10^6 parasites) are also mentioned for comparing columns. The sums of individual lipid species in *panel B* and *D* are less than the total lipid amount in *panel A* and *C* because only major species are shown. Note that the reduction of PtdSer species in the *off-state* parental strain (+ATc) is relatively negligible even though they appear somewhat blue. Nomenclature (*m:n*) is shown, where *m* and *n* signify the number of carbons and the number of double bonds in both acyl chains, respectively. All values show mean values with SEM from six independent assays (*p < 0.05, **p < 0.01, ***p < 0.001).

3.2.5. Loss of *Tg*CDS2 results in selective impairment of PtdGro synthesis

Having evaluated the impact of *Tg*CDS1 knockdown in the single mutant, we examined a role of *Tg*CDS2 for phospholipid biogenesis and functional interrelationship of both enzymes using the Δ*tgcds1$_r$*/Δ*tgcds2* strain. In essence, biochemical phenotypes of the double mutant were similar to the parental and Δ*tgcds1$_r$* strains with few noteworthy exceptions. One of the most evident effects was >80% reduction in PtdGro biogenesis upon ablation of *Tg*CDS2 (Figure 14E). All species of PtdGro were significantly decreased irrespective of ATc treatment, which confirmed a need of *Tg*CDS2 (but not *Tg*CDS1) for PtdGro biogenesis (Figure 14F). Consistently, the most common PtdGro species with short saturated fatty acids (30:0) was increased when *Tg*CDS1 was repressed in the Δ*tgcds1$_r$* strain (Figure 14F), suggesting utilization of subsequently surplus PtdOH species into PtdGro *via* *Tg*CDS2. Cardiolipin was detectable only occasionally and in minor amounts even in the parental strain; we were therefore unable to reproducibly quantify and compare its levels across the parasite strains.

The deletion of *Tg*CDS2 in the Δ*tgcds1$_r$* strain also resulted in an apparent diminution of certain PtdIns (34:2, 30:0) and PtdSer (34:2) species in the Δ*tgcds1$_r$*/Δ*tgcds2* mutant during *on state*, which were further reduced by deficiency of *Tg*CDS1 in *off state* (Figure 14B and 14D). In contrast to PtdGro, PtdIns and PtdSer, other major lipids (Appendix 7) were largely unaffected by deletion of *Tg*CDS2 when compared to the parental strain.

3.2.6. The CDS mutants show a defective growth due to impaired replication

We next determined the phenotypic effects of *Tg*CDS1 knockdown and *Tg*CDS2 knockout on the lytic cycle of tachyzoites in HFF cells. To measure the growth fitness, we first performed plaque assays, which recapitulate the consecutive lytic cycles of tachyzoites (Figure 15A). The two mutants along with the parental strain were cultured in the absence or presence of ATc. The Δ*tgcds1$_r$* strain demonstrated a normal growth during *on state*, whereas plaque size was reduced to 25% in *off state* when compared to the parental strain (Figure 15B). Plaque formation of Δ*tgcds1$_r$*/Δ*tgcds2* strain declined by a modest 12% during the *on state* with respect to the parental strain, and was severely reduced by about 90% when the expression of *Tg*CDS1 was turned off (Figure 15B). As expected, plaque numbers of both Δ*tgcds1$_r$* and Δ*tgcds1$_r$*/Δ*tgcds2* strains were similar to the parental strain in *on state*, while they dropped down to about 60% and 40% during *off state*, respectively (Figure 15C). The addition of exogenous CDP-DAG, PtdIns, PtdGro and cardiolipin to the plaque cultures were unable to restore the growth of either of the mutants (not shown), indicating that tachyzoites are unable to scavenge these lipids from the surrounding milieu.

Similar phenotype of the mutants was observed in yield assays when parasites were syringe-released and counted 40 hours post-infection (Figure 15D). As expected by aforementioned roles of CDP-DAG in membrane biogenesis, when compared to the parental strain, the parasite yield dropped by 75%, 23% and 96% after the loss of *Tg*CDS1 (*off-state* Δ*tgcds1$_r$*), *Tg*CDS2 (*on-state* Δ*tgcds1$_r$*/Δ*tgcds2*) alone or both of them (*off-state* Δ*tgcds1$_r$*/Δ*tgcds2*), respectively. Based on parasite yields, we also calculated the replication rate of all strains. The doubling time of the parental strain was about 9.4 h, which was prolonged to 16.2 h when *Tg*CDS1 expression was knocked down in the Δ*tgcds1$_r$* mutant. Knockout of *Tg*CDS2 alone caused only a modest delay in cell division (10.2 h). However, a loss of both enzymes severely attenuated the parasite proliferation (doubling time, 56.5 h). In brief, yield assays correlated well with the plaque growth of the mutants. We also assessed the replication by numerating parasites in the parasitophorous vacuoles (Figure 15F). We found an evident downshift in the vacuole sizes of both mutants following treatment with ATc. In other words, mutants in the *off state* showed a higher proportion of

smaller vacuole and *vice versa*. On contrary, both mutants showed a normal invasion rate despite ATc treatment compared to the parental strain (Fig 15E).

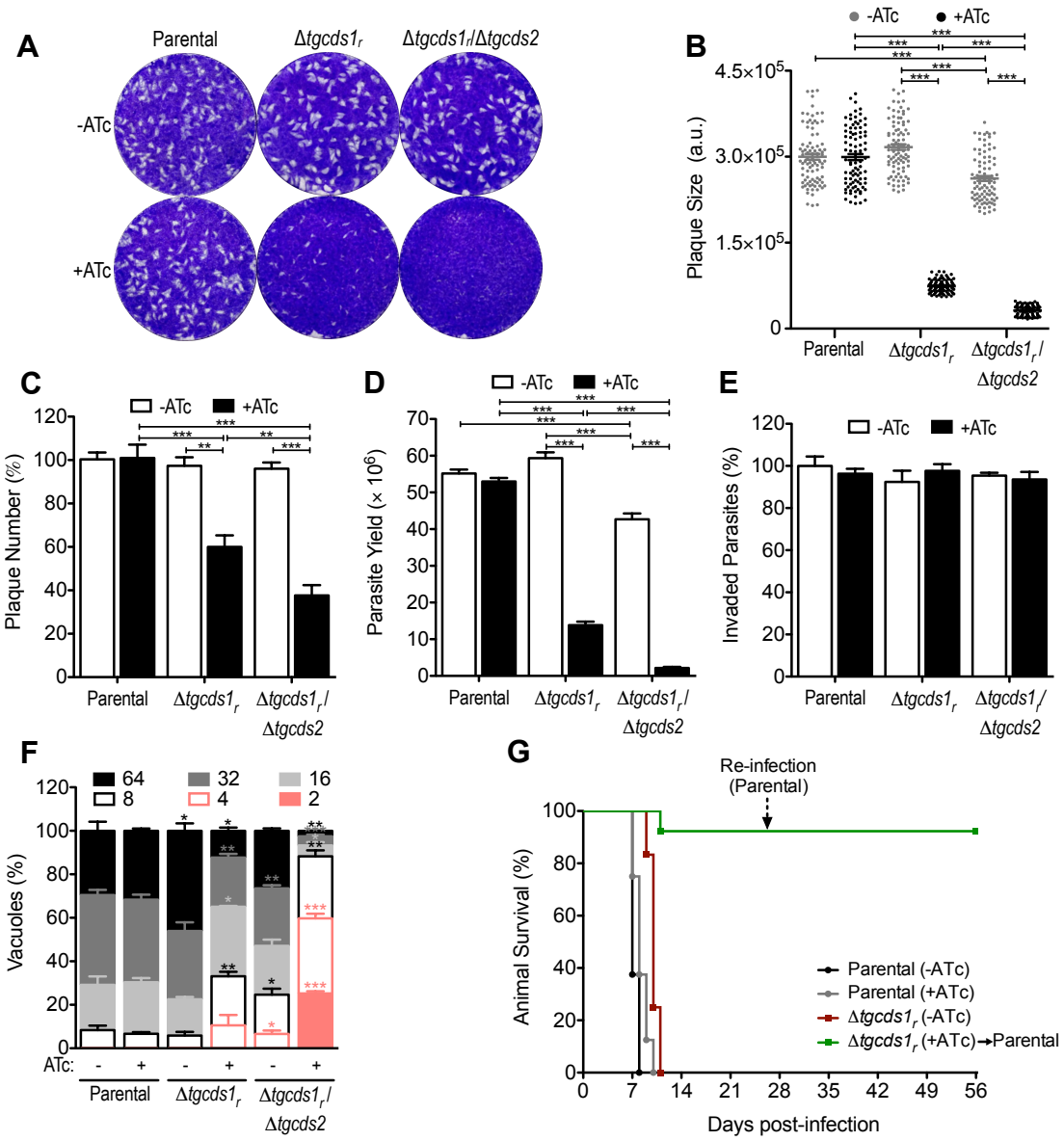


Figure 15. The Δ*tgcds1*$_r$ and Δ*tgcds1*$_r$/Δ*tgcds2* mutants show an attenuated growth. (**A**) Plaque assays using the indicated parasite strains cultured in the presence or absence of ATc. HFF monolayers were infected with 250 tachyzoites (pre-cultured with or without ATc for 4 days) and incubated for 1 week without any perturbation to allow plaque formation. (**B**) Plaque sizes of the parasite strains from *panel A*. 90 plaques of each strain were measured for area using the ImageJ software. (**C**) Plaque numbers of individual strains from *panel A*. (**D**) Yield assays in the presence or absence of ATc. Host cells were infected with 3 million tachyzoites pre-treated with ATc for 4 days before the assay (MOI, 3). Parasites were syringe-released 40 h post-infection for counting. (**E**) Invasion assays in HFFs (MOI, 10). Cells were fixed 1 h post-infection. The invasion rates of 1500-2500 parasites of each strain were presented. (**F**) Replication assays in HFFs (MOI, 1). Cells were fixed 40 h post-infection and stained with anti-GAP45 antibody to visualize intracellular tachyzoites. A total of 100-150 vacuoles were analyzed for each strain. The percentages of vacuoles containing different numbers of parasites are shown. Values are means with SEM for three (B, C, E, F) or six (D) independent experiments (*p < 0.05, **p < 0.01, ***p < 0.001). (**G**) Virulence assay in a mouse model. Animals (C57BL/6J) were infected intraperitoneally with tachyzoites (10^3) of the parental or Δ*tgcds1*$_r$ strains (pre-cultured with or without ATc for 6 days). Drug treatment during infection (2 weeks) was performed *via* the drinking water. Infected mice were monitored for 28 days (2 assays, each with 4 mice for the parental strain and 6 mice for the Δ*tgcds1*$_r$ strain). Animals surviving infection with the mutant strain (+ATc) were challenged with the parental strain (10^3) and examined for additional 28 days.

3.2.7. A deficiency of CDP-DAG synthesis attenuates the parasite virulence

Finally, we tested the physiological relevance of CDP-DAG synthesis within the parasite for virulence in a mouse model (Figure 15G). In this regard, we employed the Δ*tgcds1*$_r$ mutant along with the parental strain. Our rationale for using only the single mutant was based on a robust phenotypic impairment observed *in vitro* (*off state*, Figure 15A-D and 15F), which should ideally translate into a strong weakening of virulence *in vivo*. Hence, a double mutant (Δ*tgcds1*$_r$/Δ*tgcds2*) with even more inhibited *in vitro* growth would be more attenuated *in vivo*. Indeed, most animals infected with the Δ*tgcds1*$_r$ strain that were treated with ATc in drinking water survived as opposed to the control parental strain that was categorically lethal irrespective of ATc treatment. The mutant in its *on-state* exhibited a lethal phenotype similar to the parental strain. Collectively, these results indicated an essential role of CDS for the parasite lytic cycle and virulence in mice. The data also suggested that the parasites are unable to salvage sufficient amount of CDP-DAG from the host to bypass an ablation of autonomous synthesis. Finally, we explored the vaccination potential of the Δ*tgcds1*$_r$ strain in animals. Notably, all mice surviving the primary infection with the mutant developed sufficient immunity to resist the subsequent lethal challenge by a hypervirulent type I strain, which illustrates the prophylactic utility of a metabolically attenuated mutant to prevent acute toxoplasmosis.

3.2.8. PtdGro-P and PtdIns are made in the mitochondrion and Golgi bodies, respectively

The CDS-dependent synthesis of PtdIns and PtdGro resonates with the occurrence of PIS and PGPS in tachyzoites. The second enzyme of PtdGro synthesis PGPP could not be identified in the parasite genome. We examined subcellular distribution of *Tg*PGPS (ToxoDB, TGGT1_246530; GenBank, KX017550) by 3'-insertional tagging (HA) of the endogenous locus in *T. gondii*. *Tg*PGPS-HA was expressed in the parasite mitochondrion, as shown by its co-staining with a *bona fide* organelle marker *Tg*F1B [66](Figure 16A). We also performed genomic tagging of the *TgPIS* locus (ToxoDB, TGGT1_207710; GenBank, KX017549) with a HA tag. However, *Tg*PIS-HA was not detectable by immunofluorescence or immunoblot assays. We therefore localized *Tg*PIS-HA by ectopic overexpression. It co-localized with a known marker of Golgi body *Tg*ERD2 [83](Figure 16B). Taken together, these results indicated

synthesis of PtdGro and PtdIns in the mitochondrion and Golgi, respectively, requiring a transfer of CDP-DAG from apicoplast and ER to the sites of phospholipid biogenesis.

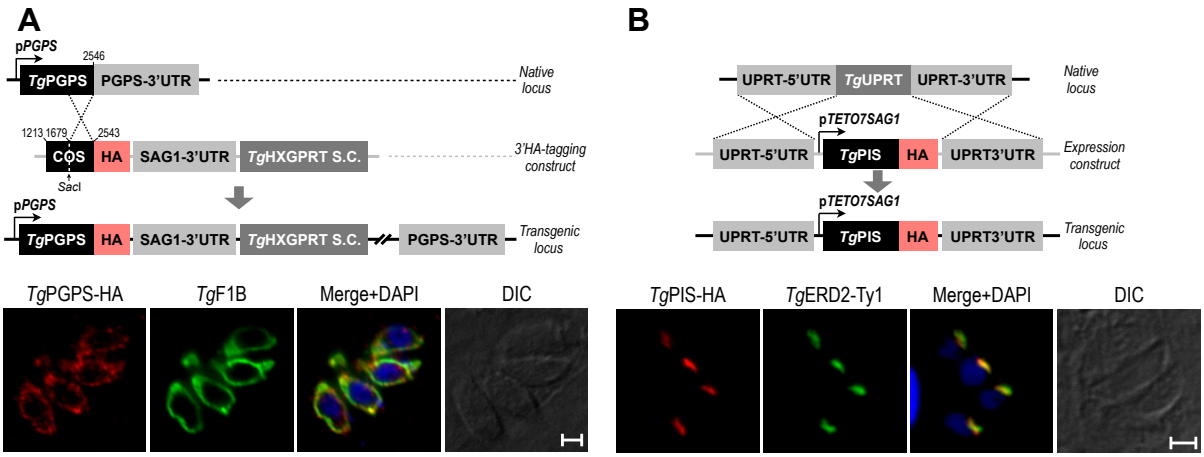


Figure 16. CDP-DAG-dependent biogenesis of PtdGro-P and PtdIns in the mitochondrion and Golgi bodies of *T. gondii* tachyzoites. Immunofluorescent images of intracellular tachyzoites expressing either (**A**) *Tg*PGPS-HA (regulated by endogenous promoter), or (**B**) *Tg*PIS-HA (under the control of p*TETO7SAG1* promoter) are shown. Indicated proteins were detected 24 h post-infection using anti-HA antibody and corresponding co-localization markers (*Tg*F1B, *Tg*ERD2-Ty1). Scale bars: 2 μm.

4. Discussion

4.1. Phospholipid synthesis in *E. falciformis* sporozoites

4.1.1. Phospholipid profile of sporozoites

Sporozoite is typically the first infective form for most apicomplexan parasites during the initial cell invasion in a new host. The process of sporogony to form sporozoites occurs in isolation from the host cells or environment. Therefore, the essential nutrients during this process, including lipids, are likely sustained solely by *de novo* synthesis, making sporozoite a unique stage to study the parasite metabolic capacity. In this study, we have examined the phospholipid content in the sporozoites of *E. falciformis*, a mouse *Eimeria* species. The detectable glycerophospholipids in sporozoites include PtdCho, PtdEtn, PtdIns, PtdSer and PtdGro. Similar compositions have been described in other protozoan parasites during various life stages, including *T. gondii* [41,45], *P. falciparum* [43,46,59,84], *T. brucei* [85-87], *T. cruzi* [88,89] and *Leishmania donovani* [90,91], confirming the role of glycerophospholipids as conserved ingredients of the parasite membranes. Additionally, we detected the presence of PtdThr in sporozoites, a rare lipid that has been recently found in *T. gondii* tachyzoites [50]. PTS enzymes could only be identified in 3 coccidian species (*E. falciformis*, *T. gondii* and *N. caninum*, Table 2), suggesting the evolution of PtdThr to facilitate a coccidian-specific role that could not be satisfied otherwise by generic lipids. Correspondingly, we show that *Ef*PTS as well as *Tg*PTS produce PtdThr to undertake the gliding motility, which in turn drives tachyzoites out of dilapidating host cells and ensuing invasion of fresh host cells. Likewise, *Ef*PTS may play a similar role in egression of sporozoites from sporocysts and invasion of epithelial cells. Interestingly, sporozoites and tachyzoites make distinct PtdThr species, which underscores mechanistic differences in the two stages.

Sphingophospholipids are another major group of phospholipids in eukaryotic cells. The main sphingophospholipid in fungi and plants is IPC [92-94], while SM and EPC are present in mammals [95]. Here we have identified IPC as the prime sphingophospholipid in *E. falciformis* sporozoites. In contrast, *T. gondii* tachyzoites possess EPC and SM, confirming previous study [45]. However, a sphingolipid synthase in *T. gondii* has been recently show to exhibit the IPC synthase activity [96],

and *in vitro* replication of tachyzoites is blocked by aureobasidin A, an inhibitor of IPC synthesis [97]. These reports indicate a trace amount of IPC is important for parasitism of *T. gondii* tachyzoites. In addition, previous studies in *T. brucei* have revealed the presence of IPC and SM in the procyclic stage of the parasite, as well as SM and EPC in the bloodstream stage [98]. Taken together, these data suggest that the sphingophospholipid biogenesis is highly stage-specific in parasites. Since IPC synthesis has no equivalent in mammalian cells, several sphingolipid synthases with IPC synthase activity have been reported as potential drug targets to treat protozoan infection [96,99,100]. However, our bioinformatics search failed to identify such enzymes in *E. falciformis* database, which may be due to incomplete annotation of the *Eimeria* genome.

The acyl chain composition determines the physical and chemical properties of lipids, and thus indicates the overall structure and function of membranes. In *Toxoplasma* and *Plasmodium*, short saturated acyl chains (C14:0 and C16:0) are produced through the FASII pathway located in the apicoplast [60,61,101]. These acyl chains are then exported to the ER, where they are modified by the FAE pathway to generate longer saturated and monounsaturated fatty acids (MUFAs) [62]. The enzymes responsible for the synthesis of polyunsaturated fatty acids (PUFAs) have not yet been identified in apicomplexan parasites. It is proposed that parasites may import PUFAs from host cells. *T. gondii* also encodes a FASI pathway; however, its function and physiological importance have not been extablished yet. In *E. falciformis* sporozoites, phospholipids are mostly composed of saturated and MUFA chains that are not longer than C18. Among them, the most abundant fatty acid is MUFA C18:1, forming C36:2 (18:1/18:1) as the dominant species for most phospholipids and C36:1 (18:0/18:1) for PtdThr (Figure 5C), similar to the fatty acid content in the oocyst walls of *E. tenella* and *Eimeria maxima* as previously reported [102]. Based on *Toxoplasma* and *Plasmodium* research, these data imply the major contributions of FASII and FAE pathways in the sporozoite stage of *E. falciformis*.

The essential roles of both FASII and FAE pathways have been demonstrated in *Toxoplasma* tachyzoites [62,103,104]. In *Plasmodium,* FASII has been proven to be crucial to merozoite maturation during liver stage but dispensable in blood stage where fatty acids are mainly scavenged from the host erythrocytes [105,106]. Additionally, FASII is essential for the sporozoite development of human *Plasmodium*

species *P. falciparum*[68,107], while in rodent *Plasmodium* species *P. berghei,* FASII is dispensable for the sporozoite formation but crucial to normal sporozoite infectivity in mice [105]. In this study, the expression of *Ef*PTS in *T. gondii* Δ*tgpts* mutant causes parasites to produce PtdThr species with tachyzoite-specific fatty acid chains (Figure 10E). Taken together, it indicates that the sources of fatty acids (whether self-biogenesis by FASII and FAE or importing from the host) are highly species- and stage-dependent, which rather than the enzyme specificity cause the different fatty acid contents between *Eimeria* sporozoites and *Toxopalsma* tachyzoites.

4.1.2. Phylogenetically divergent pathways of lipid synthesis

After fatty acids are produced in the apicoplast and ER, they are utilized with Glycerol-3P to form Lyso-PtdOH by G3PAT. We identified a fungi-type *Ef*G3PAT1 in the ER and an algae-type *Ef*G3PAT2 in the apicoplast of *E. falciformis*. This is cohesive with recent reports of their homologues in *Toxoplasma* and *Plasmodium* [63,67,69,108]. Similar to the FASII pathway, the apico-G3PAT in rodent *Plasmodium* species was found to be essential for merozoite differentiation in the liver stage and normal sporozoite infectivity *in vivo* but dispensable in the blood stage [67,69]. However, the liver stage phenotype of the apico-G3PAT mutant exhibited only partial phenocopy of the FASII mutant [69], indicating the contribution of limited Lyso-PtdOH from the ER-G3PAT. In addition, the essential algae-type apico-G3PAT in *T. gondii* is found to be responsible for the synthesis of Lyso-PtdOH (C14:0) using the short fatty acids from FASII pathway [63], while the fungi-type ER-G3PAT in *P. falciparum* displays a major preference for C16:0 and C16:1 [108]. Together with our data, it indicates that the Lyso-PtdOH production in apicomplexans is dependent on two G3PATs in the apicoplast and ER with different phylogenetic origins and fatty acid preference (Figure 17).

E. falciformis possesses two paralogs of both LPAAT and CDS for downstream PtdOH and CDP-DAG synthesis. The eukaryotic-type LPAATs and CDSs, including *Ef*LPAAT1 and *Ef*CDS1, exist in the ER or ER/Golgi complex in all protozoan parasites according to this study and previous reports [67,109-111]. On the other hand, the homologues of algae-type *Ef*LPAAT2 and prokaryotic-type *Ef*CDS2 are only present in selected protozoans (Table 2), indicating these two enzymes are lost during the evolution of other parasites. *Ef*CDS2 is localized in the apicoplast as

expected, while *Ef*LPAAT2 is found surprisingly in the ER. The results reveal an universal ER-pathway and an incomplete apicoplast pathway cooperate for the biogenesis of lipid precursors PtdOH and CDP-DAG, which requires the transfers of Lyso-PtdOH and PtdOH between apicoplast and ER/Golgi complex (Figure 17).

4.1.3. Inter-organelle cooperation for lipid biogenesis

In eukaryotic cells, lipid biosynthesis pathways are highly compartmentalized, and thus define distinct membrane characters of each organelle and their different functions. In this study, we have revealed a similar level of complexity in phospholipid synthesis network of *E. falciformis* sporozoites, however, with notable exceptions (Figure 17). We identify *Ef*CEPT1 and *Ef*CEPT2 as the last step of Kennedy pathways to synthesize major lipids PtdCho and PtdEtn, as well as *Ef*PIS for PtdIns production, in Golgi bodies. CEPT is previously reported in the ER of *P. berghei* [56] and found essential for the parasite development in *Plasmodium* blood stage [56] and *Trypanosoma* procyclic form [112]. PIS is localized in both ER and Golgi bodies of *T. brucei* and indispensable for glycosylphosphatidylinositol (GPI) synthesis and survival of the bloodstream form [113]. The occurrence of these two pathways in the Golgi bodies of *E. falciformis* endows this organelle with a significant role in lipid biogenesis of this parasite.

Two alternative pathways for PtdEtn synthesis, *Ef*PSD1 and *Ef*PSD2, are found in the mitochondrion and DG/PV of *E. falciformis*. The localizations of these two PSDs are the same as their homologues in *T. gondii* [65,66], but is different from the ER-residing PSD in *P. falciparum* [114]. Unlike in kinetoplastids where the Kennedy pathways are the main source for PtdEtn [112,115], PSD activities appear to contribute for the major PtdEtn biogenesis in apicomplexans [55,65,66]. The mitochondrion also harbors *Ef*PGPS and *Ef*CLS for the synthesis of PtdGro and cardiolipin. Similar results have been reported in *T. brucei*, where PGPS and CLS form a large protein complex on the inner mitochondrial membrane and are both essential for the mitochondrial morphology and parasite survival [116,117]. Additionally, we identify *Ef*PTS and *Ef*PSS in the ER and ER/Golgi complex, respectively, which resembles *T. gondii* [50]. Last but not least, the enzymes catalyzing the inter-conversion between PtdOH and DAG are found in the parasite periphery (*Ef*DGK1) and DG/PV (*Ef*DGK2), or with punctate intracellular distribution (*Ef*PAP). In *T. gondii*, the balance between PtdOH

and DAG is achieved by *Tg*DGK1, which is critical to the microneme secretion [118]. Such a complex network of glycerophospholipids in *E. falciformis* is expected to require a significant inter-organelle trafficking of lipids and their precursors.

Several lipid trafficking mechanisms have been reported, especially in yeast and mammalian cells, which involve lateral and transbilayer movements within the same organelle, as well as membrane contact sites, vesicular trafficking and protein-mediated transport between organelles [119-122]. In apicomplexan parasites, multiple membrane contact sites have been observed between the ER/Golgi complex, apicoplast and mitochondrion, and predicted to have important metabolic roles [123-126]. In addition, some lipid transporters have been recently identified in *Plasmodium* to transfer lipids between membranes [127,128]. However, whether they serve as privileged mechanisms of inter-organelle lipid exchange merits further investigation.

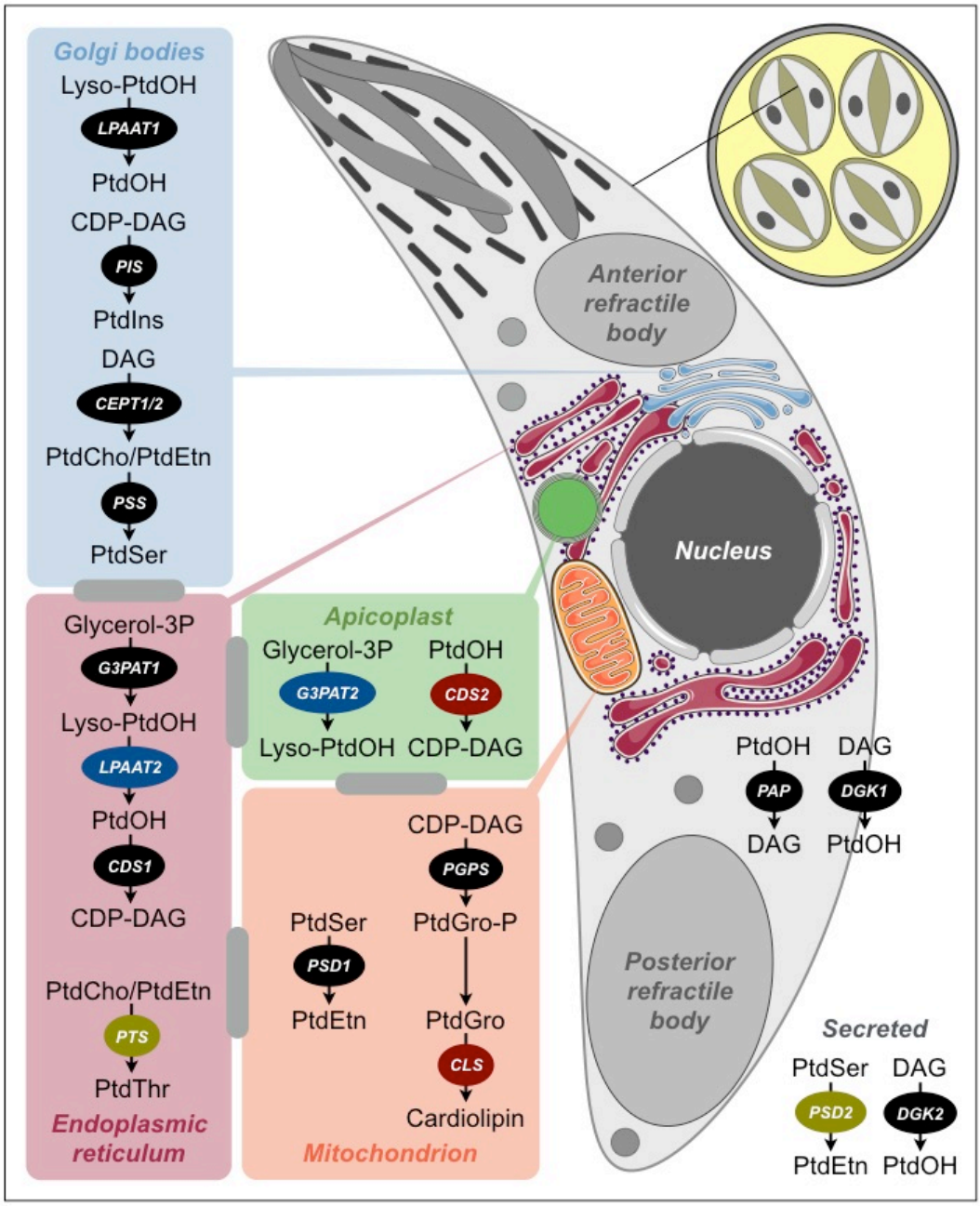


Figure 17. Model of highly compartmentalized network of phospholipid biogenesis in *E. falciformis* sporozoites. The illustration reveals the pathways that have been predicted based on subcellular localizations of the enzymes (italic with black background) in this study. Shown on the upper right corner is a sporulated oocyst enclosing 4 sporocysts, each with 2 sporozoites. Pathways located in individual organelles are zoomed out on the left. The grey bars between indicated organelles show possible inter-organelle trafficking of phospholipids and precursors. The prokaryotic-type (*Ef*CDS2 and *Ef*CLS), algal-type (*Ef*G3PAT2 and *Ef*LPAAT2) and coccidian-specific (*Ef*PTS and *Ef*PSD2) enzymes are in red, blue and yellow backgrounds, respectively. Note that *Ef*LPAAT1 and *Ef*PSS also reveal weak ER signal in addition to the dominant Golgi localization, as shown in Figure 8. *Abbreviations*: CDP-DAG, cytidine diphosphate-diacylglycerol; CDS, cytidine diphosphate-diacylglycerol synthase; CEPT, choline/ethanolamine phosphotransferase; CLS, cardiolipin synthase; DAG, diacylglycerol; DGK, diacylglycerol kinase; G3PAT, glycerol 3-phosphate acyltransferase; Glycerol-3P, glycerol 3-phosphate; LPAAT, lysophosphatidic acid acyltransferase; Lyso-PtdOH, lysophosphatidic acid; PAP, phosphatidic acid phosphatase; PGPS, phosphatidylglycerol phosphate synthase; PIS, phosphatidylinositol synthase; PSD, phosphatidylserine decarboxylase; PSS, phosphatidylserine synthase; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; PtdGro, phosphatidylglycerol; PtdGro-P, phosphatidylglycerol phosphate; PtdIns, phosphatidylinositol; PtdOH, phosphatidic acid; PtdSer, phosphatidylserine; PtdThr, phosphatidylthreonine; PTS, phosphatidylthreonine synthase.

4.2. Biogenesis and metabolic roles of CDP-DAG in *T. gondii* tachyzoites

4.2.1. Origins and subcellular localizations of CDS enzymes

CDP-DAG serves as one of the central lipid precursors in both eukaryotes and prokaryotes. Similar to what has been described in *E. falciformis* in this study, we have identified two phylogenetically distinct enzymes involved in the synthesis of CDP-DAG in *T. gondii*. *Tg*CDS1 belongs to the eukaryotic-type CDSs that are conserved across the eukaryotic organisms. It is compartmentalized in the ER of tachyzoites, similar to what has been reported for CDSs in yeast [129], plants [130] and animals [131,132]. In protozoan parasites *P. falciparum* and *T. brucei*, only one eukaryotic-type CDS has been identified, which is required for the biosynthesis of PtdIns and its descendant lipid GPI [109-111]. In mammals and plants, there are two or more eukaryotic-type CDS enzymes with different expression patterns, all of which influence PtdIns and PtdOH levels [130,133]. Interestingly, a mitochondrial maintenance protein lacking the typical cytidylyltransferase domain and CDS signature motif (Tam41) was found to catalyze the formation of CDP-DAG from PtdOH in the yeast mitochondria [134]; however, similar proteins have not been identified in any protozoan parasites. Instead, we have discovered a prokaryotic-type *Tg*CDS2 in *T. gondii* and its orthologs in only selected protozoan parasites (Table 2), suggesting a loss of the prokaryotic-type CDS during the evolution of certain parasites. *Tg*CDS2 localizes in the apicoplast, a unique plastid-like organelle evolved by two successive endosymbiotic events, first involving cyanobacteria and then red alga [135]. Both events were ensued by horizontal gene transfer from the cyanobacterial genome to the algal genome, and subsequently to the genome of the parasites. As a result, most of the apicoplast proteins, including *Tg*CDS2, have prokaryotic and/or algal origin but encoded by the parasite nucleus [135,136].

Endosymbiotic gene transfer from the apicoplast to the nucleus enables parasites to control the foreign organelle; however it requires post-translational targeting of the proteins back to the apicoplast. For many apicoplast-resident proteins, this process is guided by a N-terminal bipartite sequence comprising a signal peptide and a transit peptide [137]. Most apicoplast proteins are first imported into the ER and then transferred directly from ER to the apicoplast either through the general secretory

pathway or *via* vesicular trafficking [138-140]. Recent studies also reveal an involvement of Golgi as a sorting point for soluble proteins destined to the apicoplast [141,142]. A few other proteins, for example the membrane-bound apicoplast phosphate transporter 1 in *T. gondii*, lack a defined bipartite peptide. Instead, a tyrosine and a glycine residue at the position 16 and 17 were found to be necessary for targeting the protein to the apicoplast [143]. Both putative signal and transit peptides were identified in the N-terminal extension of *Tg*CDS2 starting from the second methionine instead of the start codon (Figure 11B and Appendix 6). Consistently, our mutagenesis studies confirm the importance of prolonged N-terminal extension and bipartite sequence for localization in the apicoplast. The data indicate that the targeting of *Tg*CDS2 to the apicoplast is mediated by the bipartite signal mechanism (Figure 12).

4.2.2. Functions of CDP-DAG in parasite biology

*Tg*CDS1 and *Tg*CDS2 mutants showed severely reduced growth and attenuated virulence, which is associated with the loss of PtdIns and PtdGro in parasites. PtdIns serves not only as a structural component of membranes, but also as a precursor for the biogenesis of some central signaling molecules. Earlier studies on the PtdIns phosphates (PIPs) have revealed that phosphatidylinositol 3-monophosphate and phosphatidylinositol (3,5)-bisphosphate are required for the apicoplast biogenesis in tachyzoites [144,145]. Another downstream product of PtdIns, GPI, has been implicated in both host cell attachment and modulation of the immune response, and proven to be crucial for the parasite survival [146-148]. On the other hand, literally nothing is known about the structural and functional relevance of PtdGro in *T. gondii*. PtdGro typically serves as an intermediate for the synthesis of cardiolipin, which is indispensable for mitochondrial homeostasis and viability of kinetoplastid parasites [116,117] but was barely detectable in tachyzoites. It is expected that changes in PtdIns, PtdGro and cardiolipin would result in organelle dysfunction and eventual demise of *T. gondii* unless parasites could salvage CDP-DAG or its descendent lipids from the environment to bypass the ablation of CDS enzymes, which appears to be not the case. A detailed morphological analysis of the CDS mutants shall reveal the underlying basis of the observed growth impairment.

*Tg*CDS1 and *Tg*CDS2 mutants did not show a perturbation of other dominant phospholipids except for PtdCho, which can be explained by the nature of underlying

pathways present in tachyzoites. We have previously reported the functional existence of PtdCho and PtdEtn syntheses through CDP-choline and CDP-ethanolamine pathways, both of which utilize DAG as the lipid backbone [55,64]. Because synthesis of PtdCho occurs exclusively through the CDP-choline route, a significant increase in the lipid content during *off state* of the two mutants is expected due to potential rerouting of PtdOH to DAG. On the other hand, PtdEtn can also be produced by decarboxylation of PtdSer in the parasite mitochondrion and DG/PV (major routes) [65,66], which may have balanced its content in the single and double mutants. PtdSer and PtdThr are generated from PtdEtn and/or PtdCho in a base-exchange manner by two distinct routes located in the parasite ER [50]. No change of PtdThr was observed in both mutants. Given its unique acyl chain composition (mostly 20:1, 20:4), it seems that a different source of glycerol backbone or lipid remodeling is required to make PtdThr. In the case of PtdSer, a sizeable increase during *on state* and decline in *off state* of both mutants was surprising and suggests the presence of yet-another PSS (CDP-DAG-dependent) for making PtdSer (Figure 18). Such a global dysregulation of major phospholipids (*e.g.*, accumulation of PtdCho and PtdOH, and/or change in PtdSer) may also underlie the phenotype observed in individual mutants. While the detection of CDP-DAG and DAG in tachyzoite is quite challenging, future studies using isotope labeling of other lipids with precursors (polar head groups or glycerol backbone) should validate aforesaid postulations.

4.2.3. A “division of labor” model of lipid biogenesis

*Tg*CDS1 and *Tg*CDS2 are utilized for the biogenesis of PtdIns, PtdSer and PtdGro in a rather selective manner. Our data suggest a model, in which ER-derived CDP-DAG fuels the synthesis of PtdIns in the Golgi bodies, whereas CDP-DAG originating in the apicoplast is utilized for PtdGro biogenesis in the mitochondrion (Figure 18). These results are consistent with the presence of two isoforms of each enzyme synthesizing Lyso-PtdOH and PtdOH, as described in *E. falciformis* in this study. One such enzyme, *Tg*G3PAT2 (termed as ATS1) has recently been reported to localize in the apicoplast [63], while others remain to be characterized. This highly spatial distribution of lipid syntheses necessitates a coordinated lipid transport among various organelles in *T. gondii*, in particular a ‘retrograde’ transfer of PtdIns from Golgi to ER, and of CDP-DAG from ER/apicoplast to Golgi/mitochondrion. This study

embodying a prototype model of lipid synthesis and trafficking provides a framework for such paradigms in a well-established model eukaryotic pathogen.

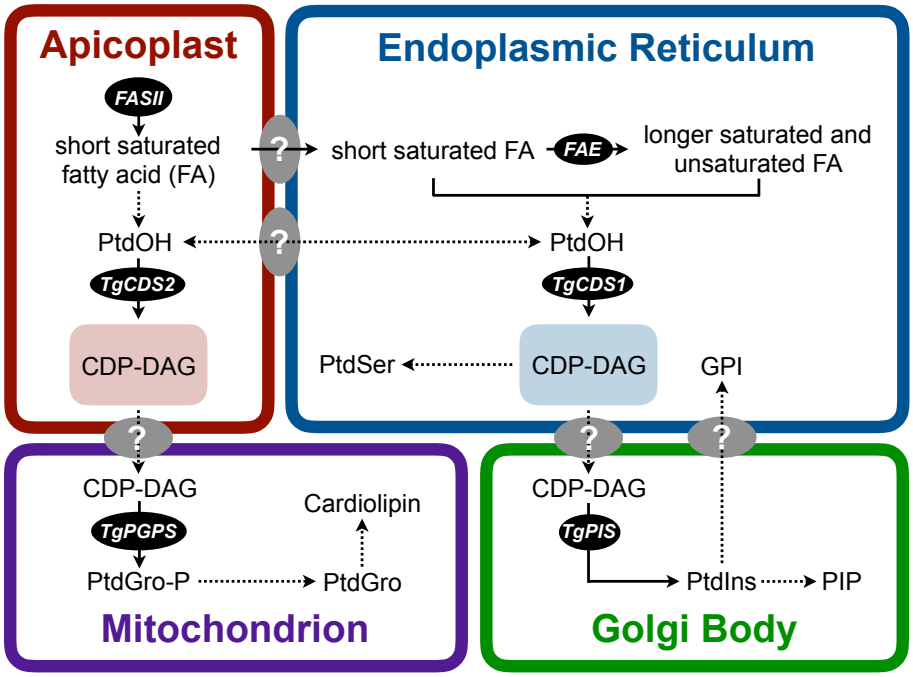


Figure 18. A "division of labor" model of CDP-DAG-dependent biogenesis of PtdIns, PtdSer and PtdGro involving the inter-organelle cooperation of specified organelles. The illustration depicts abridged inter-organelle trafficking of only selected lipids, especially those relevant to the context. Many other possibilities also exist. Two distinct CDP-DAG pools are utilized to produce PtdIns, PtdSer and PtdGro. The CDP-DAG pool in the ER is generated by *Tg*CDS1 and used for biogenesis of PtdIns in the Golgi body. PtdIns could subsequently be used to synthesize phosphatidylinositol phosphates (PIP) and glycosylphosphatidylinositol (GPI). *Tg*CDS2 generates a second pool of CDP-DAG in the apicoplast that contributes to syntheses of PtdGro phosphate (PtdGro-P), PtdGro and cardiolipin in the mitochondrion. Pathways that have been confirmed are marked with solid line arrows, while pathways and transports in postulation are marked with dashed line arrows.

5. Conclusions and perspectives

This work reveals distinct phospholipid profiles of the infective sporozoite and tachyzoite forms of apicomplexan parasites *E. falciformis* and *T. gondii*. We also demonstrate a highly compartmentalized network of parasite lipid biogenesis, involving significant inter-organelle cooperation and lipid trafficking. The enzymes in this network are evolved from various origins including both prokaryotes and eukaryotes. Our work establishes a “division of labor” model of lipid biogenesis in apicomplexan parasites. Natural occurrence of atypical lipids, dependence on *de novo* synthesis and evolutionary divergence of certain pathways together offer unique opportunities to selectively inhibit parasite development and thereby prevent inter-host transmission.

Many questions still remain to be answered in future study, including:

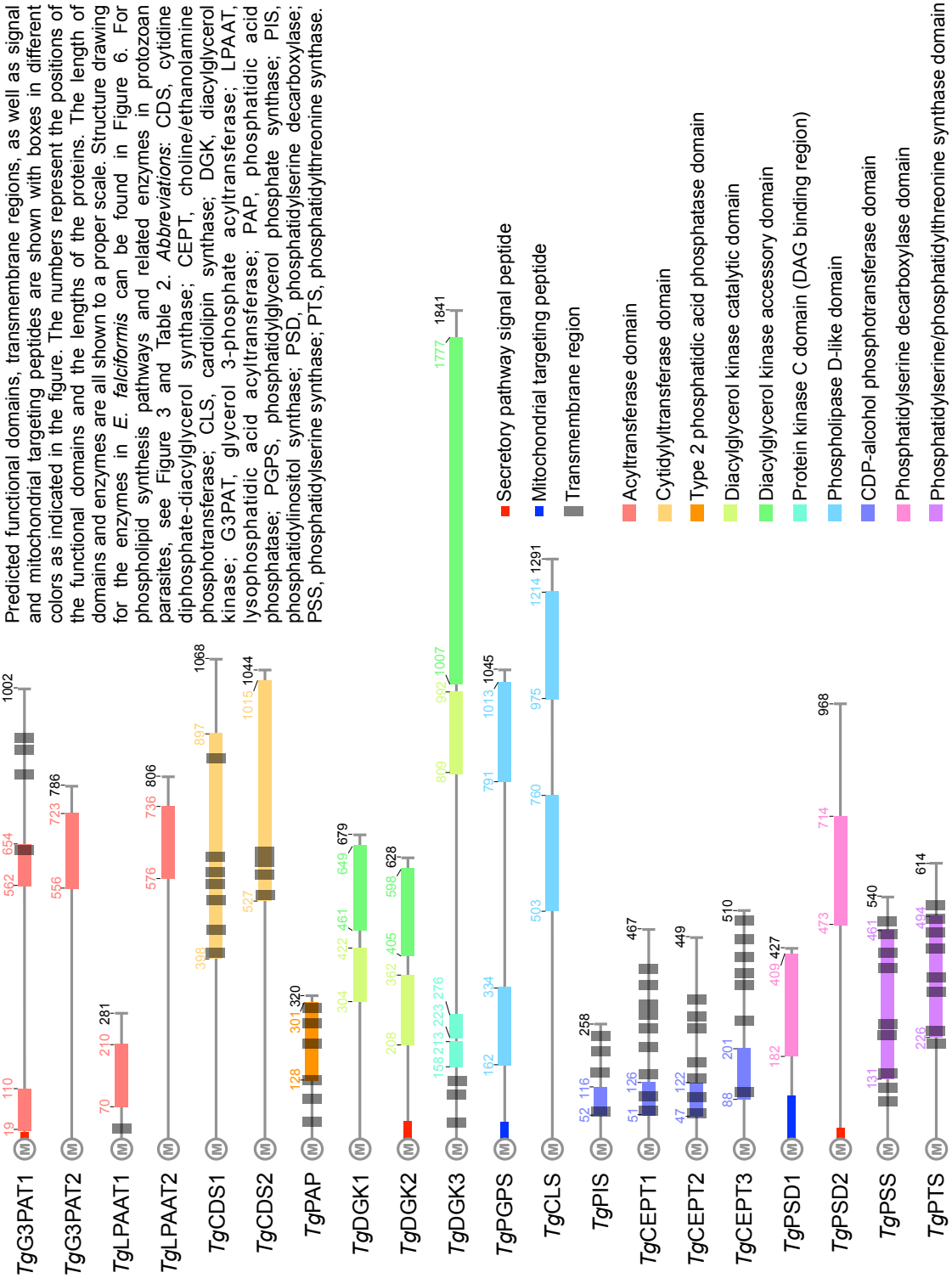
- Synthesis of IPC, PtdIns, GPI and cardiolipin;
- Lipid compositions of individual organelles;
- Mechnism of inter-organelle lipid trafficking;
- Genetic ablation of lipid synthesis in *E. falciformis*;
- Roles of lipids during sexual development of parasites using *E. falciformis* as a model.

Appendices

Appendix 1. List of sequences from *E. falciformis* and *T. gondii* deposited to GenBank in this study.

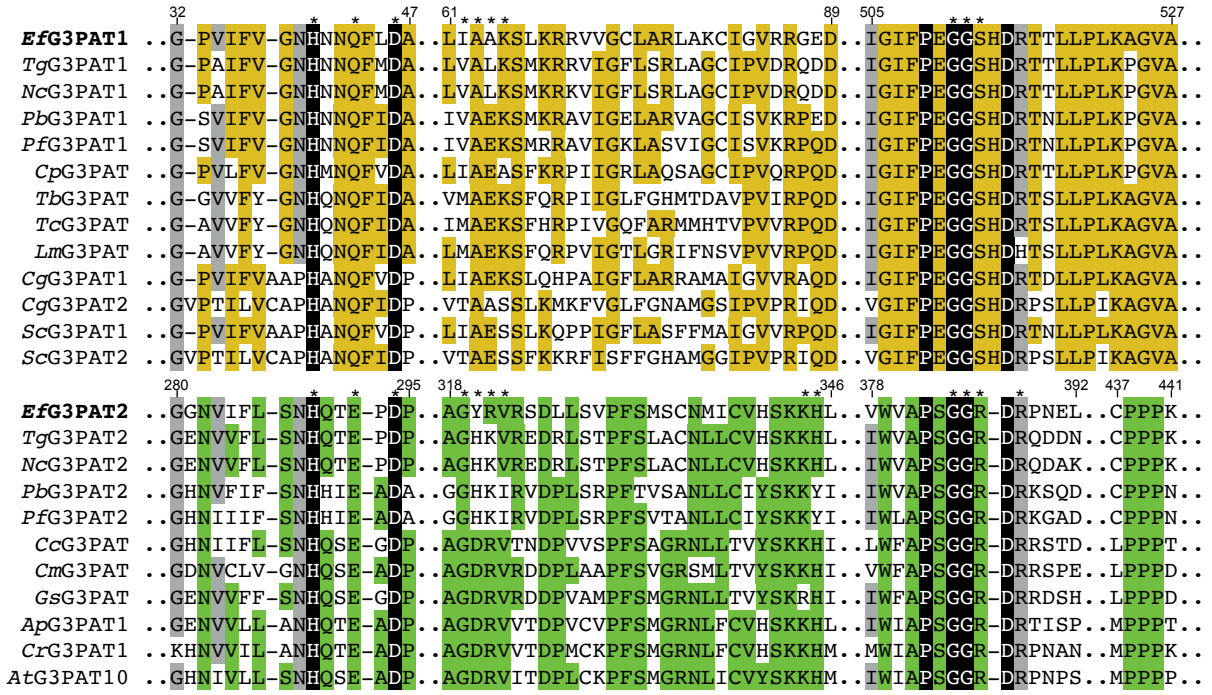
Gene name	EuPathDB accession number	Genbank accession number
*Ef*G3PAT1	EfaB_PLUS_45924.g2677	KX785365
*Ef*G3PAT2	EfaB_MINUS_3469.g362	KX785366
*Ef*LPAAT1	EfaB_MINUS_17400.g1567	KX785367
*Ef*LPAAT2	EfaB_MINUS_12065.g1058	KX785368
*Ef*CDS1	EfaB_PLUS_1048.g115	KX017547
*Ef*CDS2	EfaB_PLUS_36188.g2485	KX017548
*Ef*PAP	EfaB_MINUS_22960.g1971	KX785369
*Ef*DGK1	EfaB_MINUS_7048.g670	KX785370
*Ef*DGK2	EfaB_MINUS_36188.g2575	KX785371
*Ef*DGK3	EfaB_MINUS_11882.g1052	KX785372
*Ef*PGPS	EfaB_MINUS_17907.g1626	KX785373
*Ef*CLS	EfaB_PLUS_56725.g2797	KX785374
*Ef*PIS	EfaB_PLUS_6035.g572	KX785375
*Ef*CEPT1	EfaB_MINUS_56725.g2919	KX785376
*Ef*CEPT2	EfaB_MINUS_15745.g1427	KX785377
*Ef*CEPT3	EfaB_MINUS_25458.g2174	KX785378
*Ef*PSD1	EfaB_MINUS_22450.g1948	KX785379
*Ef*PSD2	EfaB_MINUS_1072.g165	KX785380
*Ef*PSS	EfaB_PLUS_1974.g212	KX785381
*Ef*PTS	EfaB_MINUS_800.g81	KX785382
*Tg*CDS1	TGGT1_281980	KU199242
*Tg*CDS2	TGGT1_263785	KU199243
*Tg*PGPS	TGGT1_246530	KX017550
*Tg*PIS	TGGT1_207710	KX017549

Appendix 2. Schematic illustration showing the primary structures of putative enzymes involved in phospholipid synthesis of *T. gondii*.

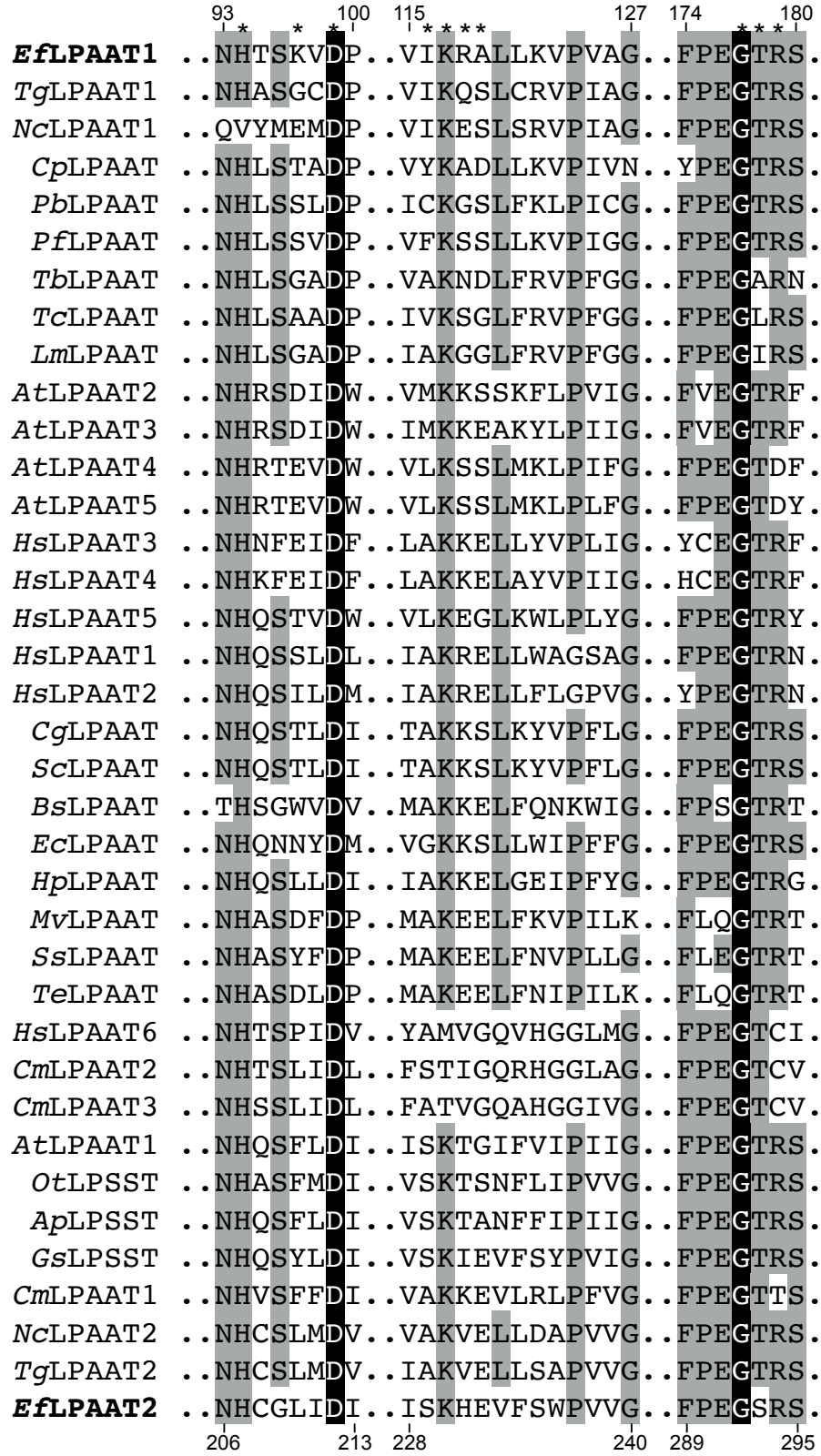


Appendix 3. Multiple alignments of the conserved functional motifs of chosen lipid synthesis enzymes from selected organisms.

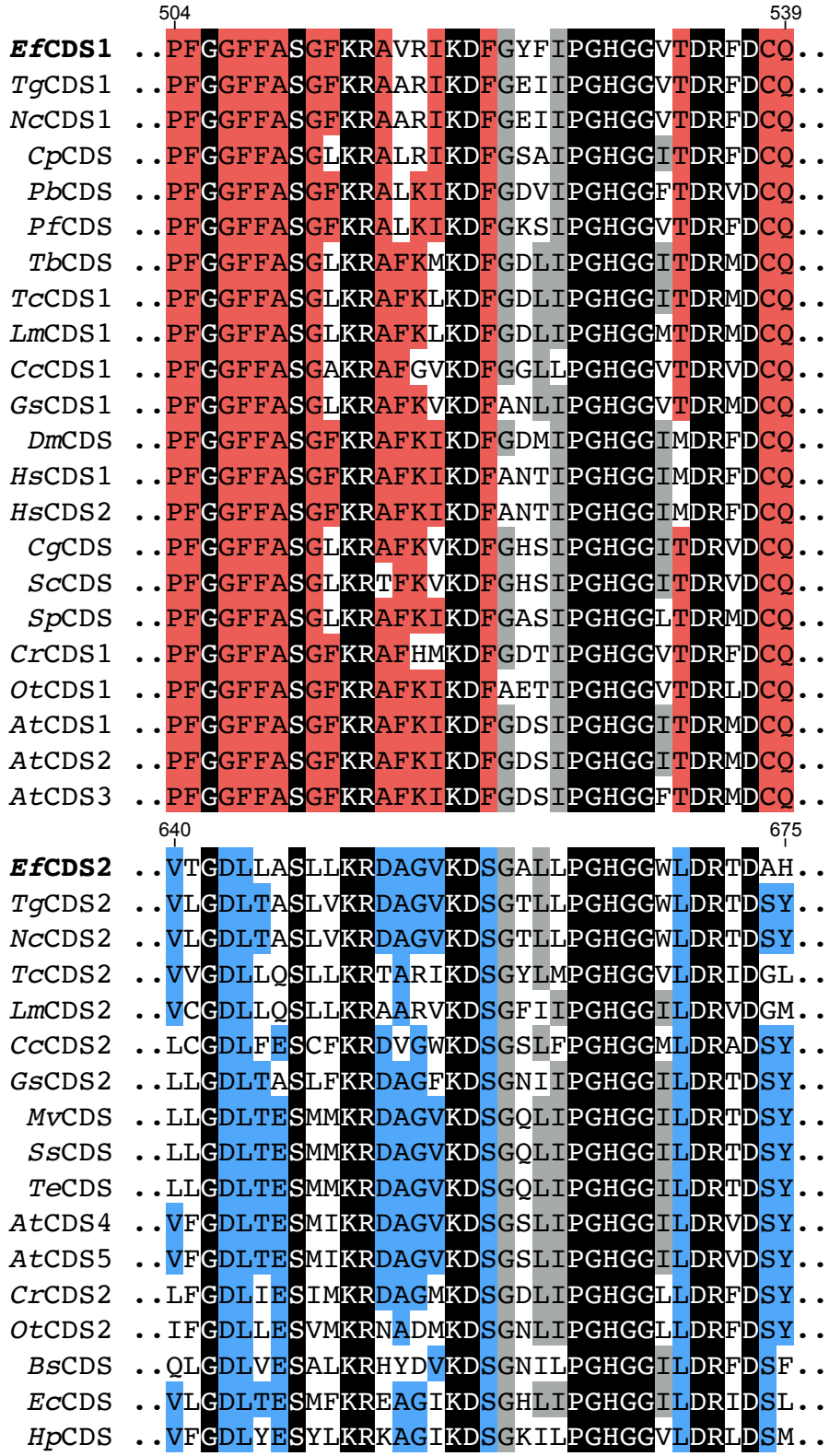
(**A**) Alignment of conserved motifs of G3PATs in the same clades with *Ef*G3PAT1 and *Ef*G3PAT2 in the phylogenetic tree (Figure 7A). The residues identical across all sequences are shaded with black color, while those identical in more than half of the sequences are shaded in grey. The residues only conserved in one clade of sequences were shaded with yellow and green, respectively. The residues contributing to acyl-acceptor binding pockets are marked with stars on top of the alignment. Sequence information including accession numbers and full organism names are shown in Appendix 5.



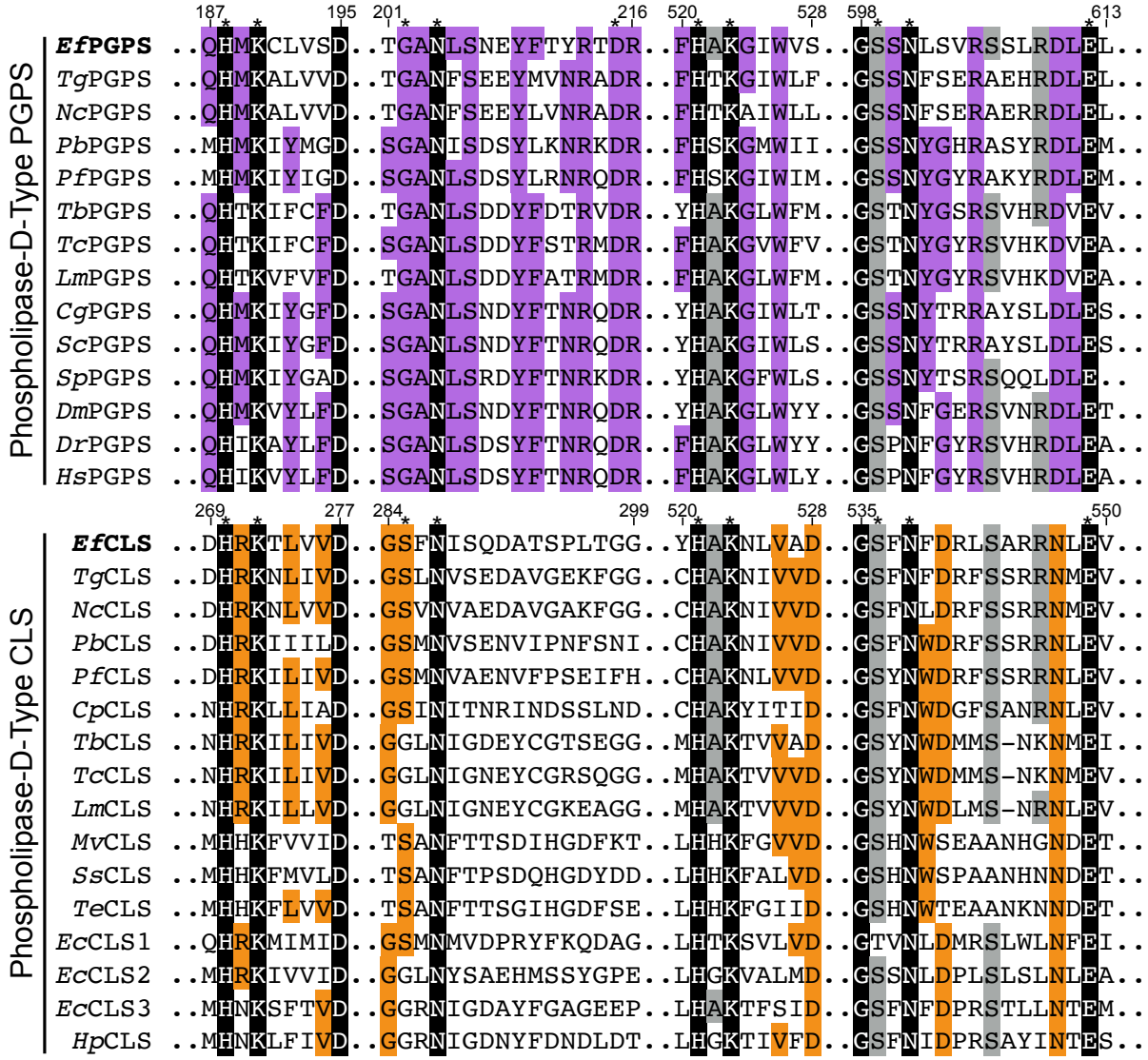
(**B**) Alignment of conserved motifs of LPAATs from selected organisms (see Figure 7B for phylogenetic tree). The residues identical across all sequences are shaded with black color, while those identical in more than half of the sequences are shaded in grey. The residues contributing to acyl-acceptor binding pockets are marked with stars on top of the alignment. Sequence information including accession numbers and full organism names are shown in Appendix 5.



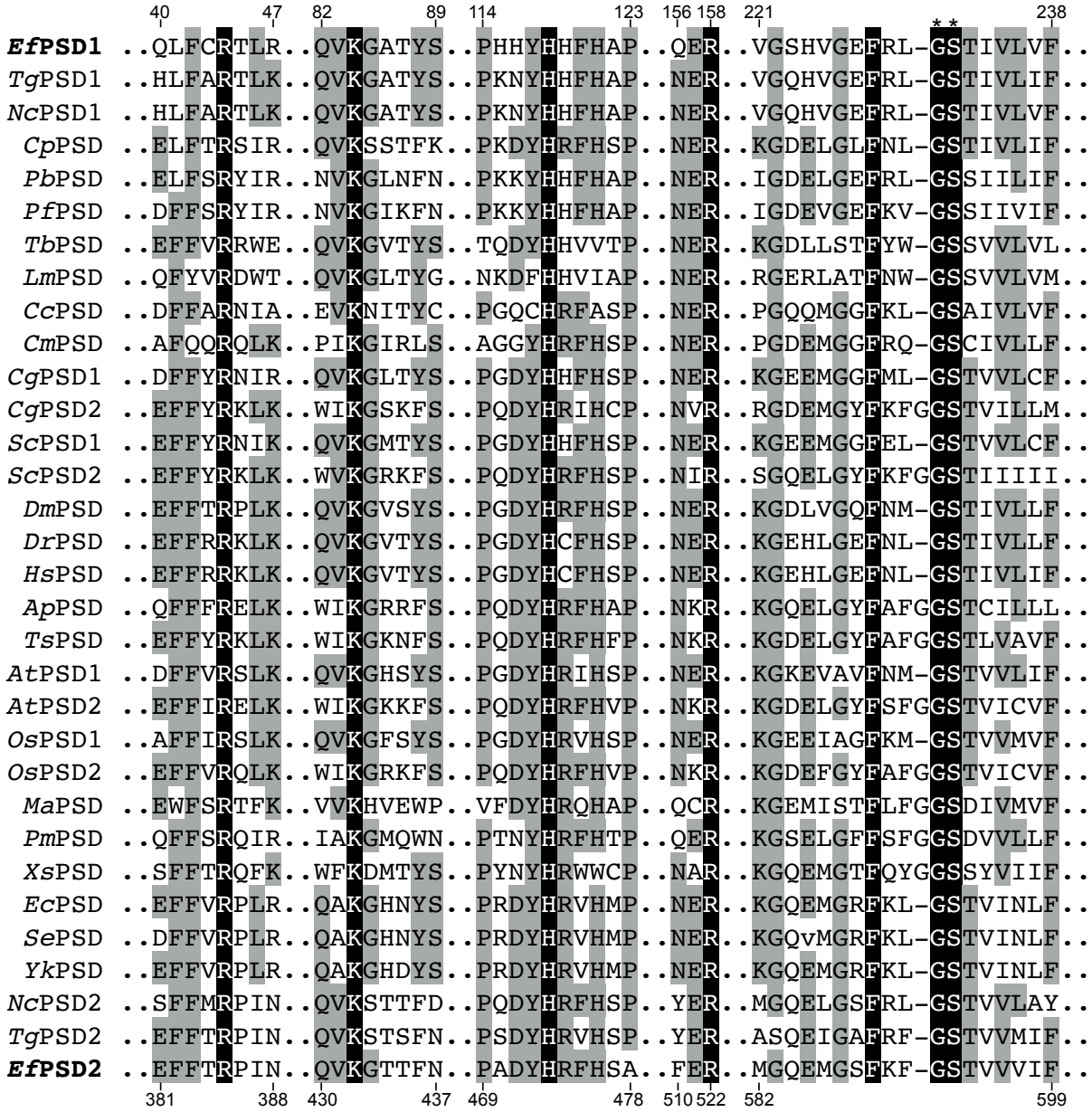
(**C**) Alignment of conserved motifs of CDSs from selected organisms (see Figure 7C and 11A for phylogenetic tree). The residues identical across all sequences are shaded with black color, while those identical in more than half of the sequences are shaded in grey. The residues only conserved in eukaryotic or prokaryotic sequences were shaded with red and blue, respectively. Sequence information including accession numbers and full organism names are shown in Appendix 5.



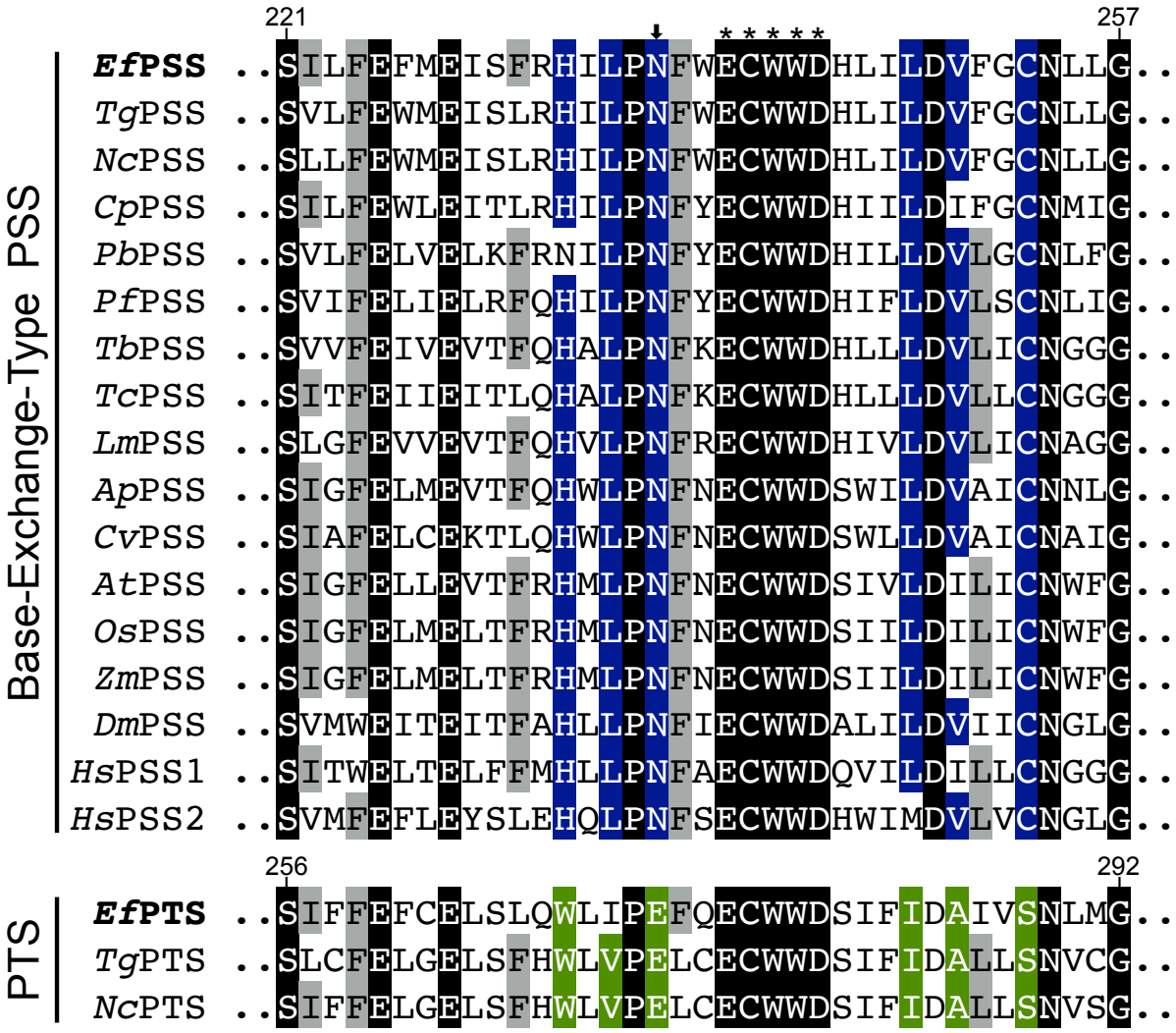
(**D**) Alignment of conserved motifs of phospholipase-D-type PGPSs and CLSs from selected organisms (see Figure 7D for phylogenetic tree). See Appendix 3I For CDP-alcohol-phosphotransferase-type PGPSs and CLSs. The residues identical across all sequences are shaded with black color, while those identical in more than half of the sequences are shaded in grey. The residues only conserved in PGPS or CLS sequences were shaded with purple and orange, respectively. The putative catalytic sites are marked with stars on top of the alignment. Sequence information including accession numbers and full organism names are shown in Appendix 5.



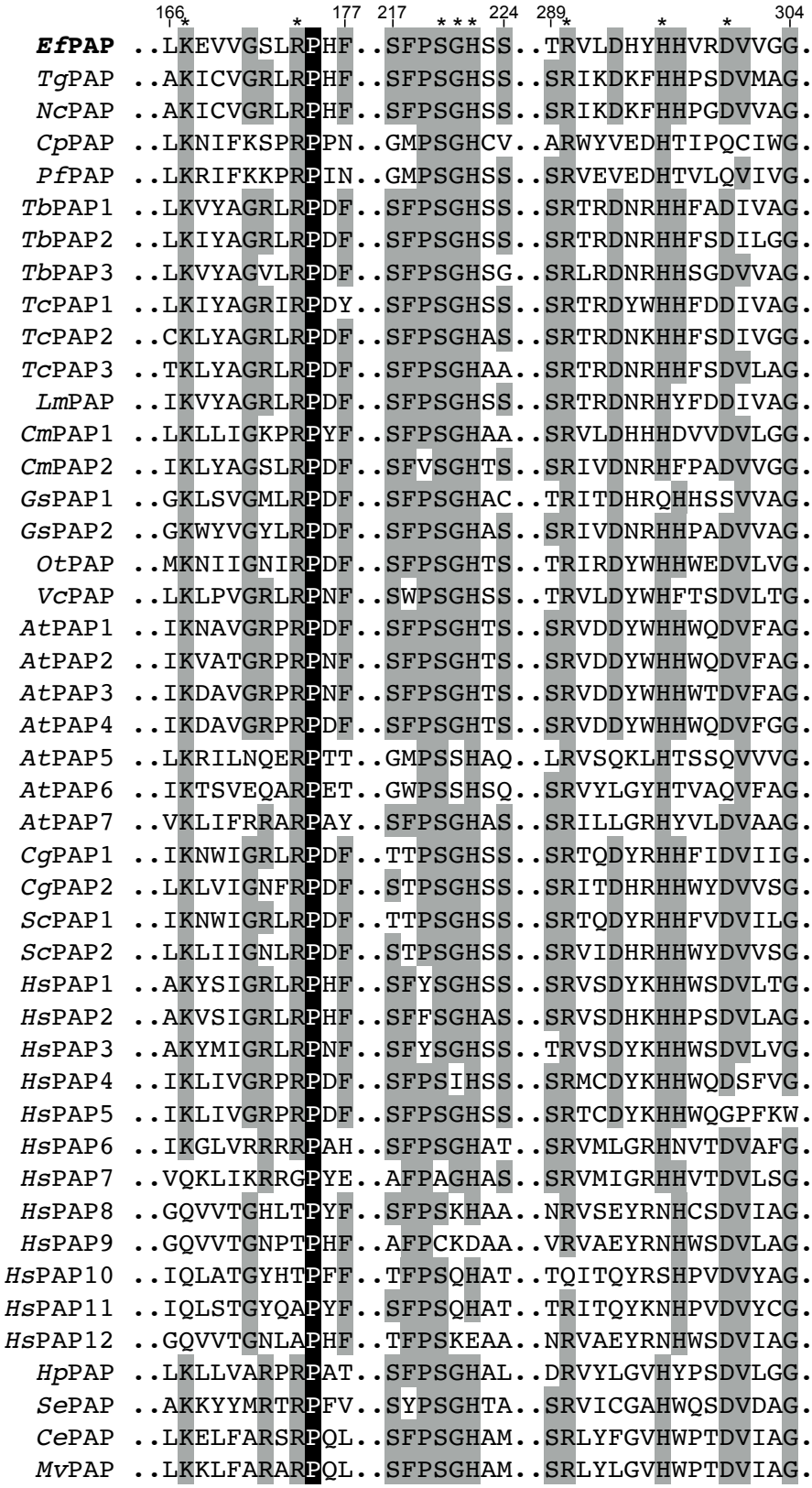
(**E**) Alignment of conserved motifs of PSDs from selected organisms (see Figure 7E for phylogenetic tree). The residues identical across all sequences are shaded with black color, while those identical in more than half of the sequences are shaded in grey. The cleavage residues at the catalytic sites are marked with stars on top of the alignment. Sequence information including accession numbers and full organism names are shown in Appendix 5.



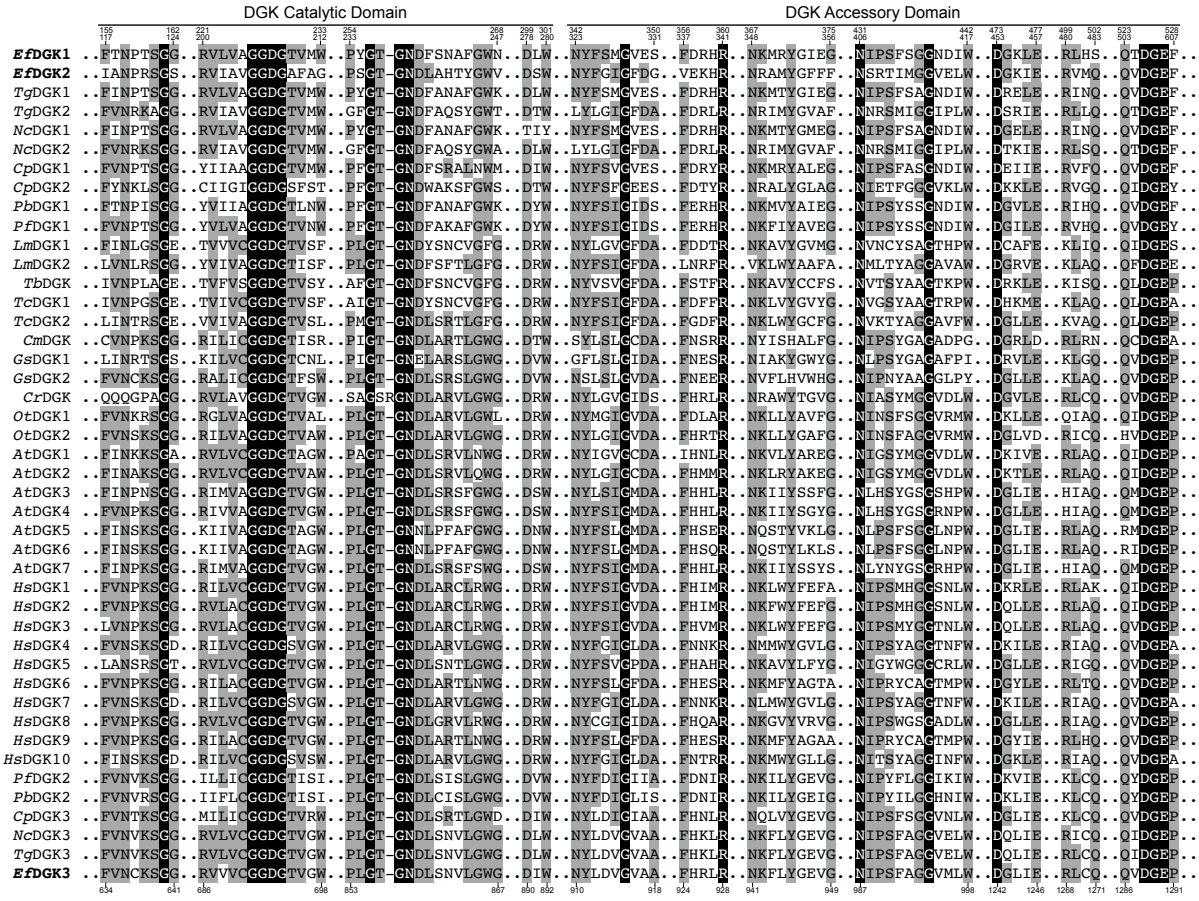
(**F**) Alignment of conserved motifs of base-exchange-type PSSs and PTSs from selected organisms (see Figure 7F for phylogenetic tree). See Appendix 3I For CDP-alcohol-phosphotransferase-type PSSs. The residues identical across all sequences are shaded with black color, while those identical in more than half of the sequences are shaded in grey. The residues only conserved in PSS or PTS sequences were shaded with blue and green, respectively. The putative catalytic site (ECWWD) is marked with stars on top of the alignment, which was deleted in the Δ*tgpts* mutant (see Figure 9A). The substrate-binding residue is highlighted with arrow. Sequence information including accession numbers and full organism names are shown in Appendix 5.



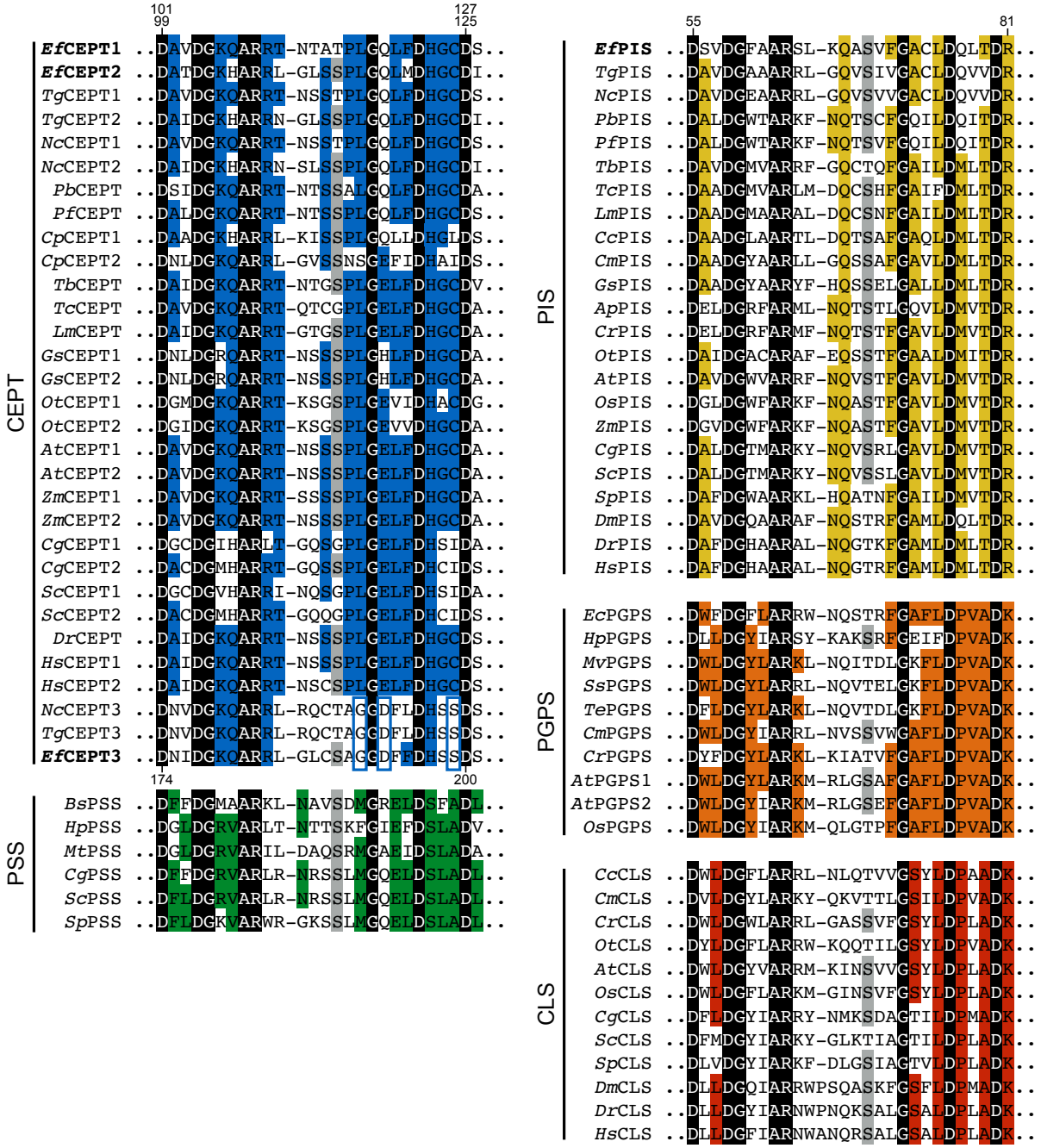
(**G**) Alignment of conserved motifs of PAPs from selected organisms (see Appendix 4A for phylogenetic tree). The residues identical across all sequences are shaded with black color, while those identical in more than half of the sequences are shaded in grey. The PAP active residues are marked with stars on top of the alignment. Sequence information including accession numbers and full organism names are shown in Appendix 5.



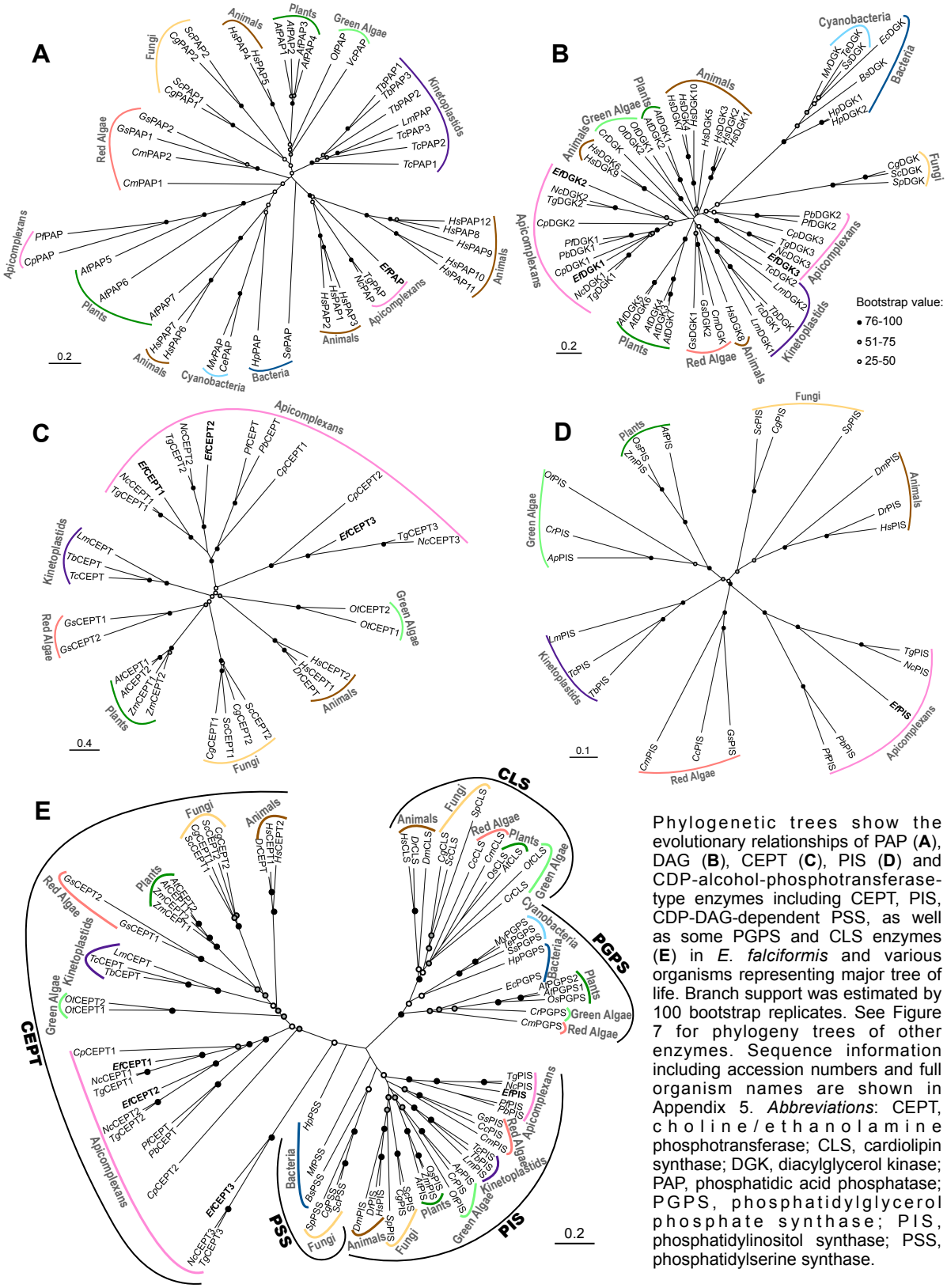
(**H**) Alignment of conserved motifs of DGKs from selected organisms (see Appendix 4B for phylogenetic tree). The residues identical across all sequences are shaded with black color, while those identical in more than half of the sequences are shaded in grey. Sequence information including accession numbers and full organism names are shown in Appendix 5.



(**I**) Alignment of conserved motifs of CDP-alcohol-phosphotransferase-type enzymes (CEPTs, PISs, CDP-DAG-dependent PSSs, as well as some PGPSs and CLSs) from selected organisms (see Figure 7D, 7F and Appendix 4C-E for phylogenetic trees). The residues identical across all sequences are shaded with black color, while those identical in more than half of the sequences are shaded in grey. The residues only conserved in one group of enzymes were shaded with different colors. The three residues only identical in CEPT3s from coccidian parasites and different from other CEPTs are highlighted with blue boxes. Sequence information including accession numbers and full organism names are shown in Appendix 5.



Appendix 4. Phylogeny of selected lipid synthesis enzymes.



Phylogenetic trees show the evolutionary relationships of PAP (**A**), DAG (**B**), CEPT (**C**), PIS (**D**) and CDP-alcohol-phosphotransferase-type enzymes including CEPT, PIS, CDP-DAG-dependent PSS, as well as some PGPS and CLS enzymes (**E**) in *E. falciformis* and various organisms representing major tree of life. Branch support was estimated by 100 bootstrap replicates. See Figure 7 for phylogeny trees of other enzymes. Sequence information including accession numbers and full organism names are shown in Appendix 5. *Abbreviations*: CEPT, choline/ethanolamine phosphotransferase; CLS, cardiolipin synthase; DGK, diacylglycerol kinase; PAP, phosphatidic acid phosphatase; PGPS, phosphatidylglycerol phosphate synthase; PIS, phosphatidylinositol synthase; PSS, phosphatidylserine synthase.

Appendix 5. Sequences used for phylogenetic analysis.

Sequences from apicomplexan and kinetoplastid parasites are shown with EuPathDB accession numbers, while the rest are provided with NCBI accession numbers. See Appendix 1 for the Genbank accession numbers of *E. falciformis* sequences.

	Designation	Species	Accession No.
Glycerol 3-Phosphate Acyltransferase (G3PAT)			
Apicomplexans	*Cp*G3PAT	*Cryptosporidium parvum*	cgd6_1270
	*Ef*G3PAT1	*Eimeria falciformis*	EfaB_PLUS_45924.g2677
	*Ef*G3PAT2	*Eimeria falciformis*	EfaB_MINUS_3469.g362
	*Nc*G3PAT1	*Neospora caninum*	NCLIV_029980
	*Nc*G3PAT2	*Neospora caninum*	NCLIV_035870
	*Pb*G3PAT1	*Plasmodium berghei*	PBANKA_1428500
	*Pb*G3PAT2	*Plasmodium berghei*	PBANKA_1416700
	*Pf*G3PAT1	*Plasmodium falciparum*	PF3D7_1212500
	*Pf*G3PAT2	*Plasmodium falciparum*	PF3D7_1318200
	*Tg*G3PAT1	*Toxoplasma gondii*	TGGT1_256980
	*Tg*G3PAT2	*Toxoplasma gondii*	TGGT1_270910
Kinetoplastids	*Lm*G3PAT	*Leishmania major*	LmjF.03.0080
	*Tb*G3PAT	*Trypanosoma brucei*	Tb927.10.3100
	*Tc*G3PAT	*Trypanosoma cruzi*	TcCLB.510943.150
Bacteria	*Bs*G3PAT	*Bacillus subtilis*	AIY97425
	*Ec*G3PAT	*Escherichia coli*	WP_001272797
	*Hp*G3PAT	*Helicobacter pylori*	ALM81555
Cyanobacteria	*Mv*G3PAT	*Microcoleus vaginatus*	EGK86078
	*Pm*G3PAT	*Prochlorococcus marinus*	KZR82092
	*Ss*G3PAT	*Synechocystis sp. PCC6803*	ALJ67680
	*Te*G3PAT	*Trichodesmium erythraeum*	WP_011612551
Red Algae	*Cc*G3PAT	*Chondrus crispus*	CDF35600
	*Cm*G3PAT	*Cyanidioschyzon merolae*	BAM80174
	*Gs*G3PAT	*Galdieria sulphuraria*	EME29189
Green Algae	*Ap*G3PAT1	*Auxenochlorella protothecoides*	XP_011399328
	*Ap*G3PAT2	*Auxenochlorella protothecoides*	KFM27021
	*Cr*G3PAT1	*Chlamydomonas reinhardtii*	EDP02129
	*Cr*G3PAT2	*Chlamydomonas reinhardtii*	AFC93411
Plants	*At*G3PAT1	*Arabidopsis thaliana*	Q9SHJ5
	*At*G3PAT2	*Arabidopsis thaliana*	Q9FZ22
	*At*G3PAT3	*Arabidopsis thaliana*	Q9SYJ2
	*At*G3PAT4	*Arabidopsis thaliana*	Q9LMM0
	*At*G3PAT5	*Arabidopsis thaliana*	Q9CAY3
	*At*G3PAT6	*Arabidopsis thaliana*	O80437
	*At*G3PAT7	*Arabidopsis thaliana*	Q9LHS7
	*At*G3PAT8	*Arabidopsis thaliana*	Q5XF03
	*At*G3PAT9	*Arabidopsis thaliana*	AED 97358
	*At*G3PAT10	*Arabidopsis thaliana*	Q43307
Fungi	*Cg*G3PAT1	*Candida glabrata*	KTB24783
	*Cg*G3PAT2	*Candida glabrata*	KTB25967
	*Sc*G3PAT1	*Saccharomyces cerevisiae*	P32784
	*Sc*G3PAT2	*Saccharomyces cerevisiae*	P36148
Animals	*Hs*G3PAT1	*Homo sapiens*	NP_065969
	*Hs*G3PAT2	*Homo sapiens*	NP_997211
	*Hs*G3PAT3	*Homo sapiens*	NP_116106
	*Hs*G3PAT4	*Homo sapiens*	NP_848934
Lysophosphatidic Acid Acyltransferase (LPAAT)			

Apicomplexans	*Cp*LPAAT	*Cryptosporidium parvum*	cgd8_1400
	*Ef*LPAAT1	*Eimeria falciformis*	EfaB_MINUS_17400.g1567
	*Ef*LPAAT2	*Eimeria falciformis*	EfaB_MINUS_12065.g1058
	*Nc*LPAAT1	*Neospora caninum*	NCLIV_017010
	*Nc*LPAAT2	*Neospora caninum*	NCLIV_006550
	*Pb*LPAAT	*Plasmodium berghei*	PBANKA_1308200
	*Pf*LPAAT	*Plasmodium falciparum*	PF3D7_1444300
	*Tg*LPAAT1	*Toxoplasma gondii*	TGGT1_240860
	*Tg*LPAAT2	*Toxoplasma gondii*	TGGT1_297640
Kinetoplastids	*Lm*LPAAT	*Leishmania major*	LmjF.32.1960
	*Tb*LPAAT	*Trypanosoma brucei*	Tb927.11.15150
	*Tc*CLPAAT	*Trypanosoma cruzi*	TcCLB.510723.29
Bacteria	*Bs*LPAAT	*Bacillus subtilis*	O07584
	*Ec*LPAAT	*Escherichia coli*	P26647
	*Hp*LPAAT	*Helicobacter pylori*	O25903
Cyanobacteria	*Mv*LPAAT	*Microcoleus vaginatus*	WP_006633731
	*Ss*LPAAT	*Synechocystis sp. PCC6803*	WP_010873224
	*Te*LPAAT	*Trichodesmium erythraeum*	WP_011611451
Red Algae	*Cm*LPAAT1	*Cyanidioschyzon merolae*	BAM82661
	*Cm*LPAAT2	*Cyanidioschyzon merolae*	BAM80575
	*Cm*LPAAT3	*Cyanidioschyzon merolae*	BAM78739
	*Gs*LPAAT	*Galdieria sulphuraria*	EME27983
Green Algae	*Ap*LPAAT	*Auxenochlorella protothecoides*	KFM23593
	*Ot*LPAAT	*Ostreococcus tauri*	CEF98793
Plants	*At*LPAAT1	*Arabidopsis thaliana*	Q8GXU8
	*At*LPAAT2	*Arabidopsis thaliana*	Q8LG50
	*At*LPAAT3	*Arabidopsis thaliana*	Q9SYC8
	*At*LPAAT4	*Arabidopsis thaliana*	Q8L4Y2
	*At*LPAAT5	*Arabidopsis thaliana*	Q9LHN4
Fungi	*Cg*LPAAT	*Candida glabrata*	KTB01584
	*Sc*LPAAT	*Saccharomyces cerevisiae*	P33333
Animals	*Hs*LPAAT1	*Homo sapiens*	NP_006402
	*Hs*LPAAT2	*Homo sapiens*	NP_006403
	*Hs*LPAAT3	*Homo sapiens*	NP_064517
	*Hs*LPAAT4	*Homo sapiens*	NP_064518
	*Hs*LPAAT5	*Homo sapiens*	NP_060831
	*Hs*LPAAT6	*Homo sapiens*	AAP21893
CDP-Diacylglycerol Synthase (CDS)			
Apicomplexans	*Cp*CDS	*Cryptosporidium parvum*	cgd7_450
	*Ef*CDS1	*Eimeria falciformis*	EfaB_PLUS_1048.g115
	*Ef*CDS2	*Eimeria falciformis*	EfaB_PLUS_36188.g2485
	*Nc*CDS1	*Neospora caninum*	NCLIV_023660
	*Nc*CDS2	*Neospora caninum*	NCLIV_024160
	*Pb*CDS	*Plasmodium berghei*	PBANKA_1032600
	*Pf*CDS	*Plasmodium falciparum*	PF3D7_1409900
	*Tg*CDS1	*Toxoplasma gondii*	TGGT1_281980
	*Tg*CDS2	*Toxoplasma gondii*	TGGT1_263785
Kinetoplastids	*Lm*CDS1	*Leishmania major*	LmjF.26.1620
	*Lm*CDS2	*Leishmania major*	LmjF.32.2870
	*Tb*CDS	*Trypanosoma brucei*	Tb927.7.220
	*Tc*CDS1	*Trypanosoma cruzi*	TcCLB.511237.40
	*Tc*CDS2	*Trypanosoma cruzi*	TcCLB.508707.140
Bacteria	*Bs*CDS	*Bacillus subtilis*	KFF57052
	*Ec*CDS	*Escherichia coli*	P0ABG1
	*Hp*CDS	*Helicobacter pylori*	WP_029567400
Cyanobacteria	*Mv*CDS	*Microcoleus vaginatus*	WP_006633099
	*Pm*CDS	*Prochlorococcus marinus*	WP_011130437

	*Ss*CDS	*Synechocystis sp. PCC6803*	P73548
	*Te*CDS	*Trichodesmium erythraeum*	WP_011611001
Red Algae	*Cc*CDS1	*Chondrus crispus*	XP_005717743
	*Cc*CDS2	*Chondrus crispus*	XP_005711550
	*Gs*CDS1	*Galdieria sulphuraria*	EME30016
	*Gs*CDS2	*Galdieria sulphuraria*	EME29594
Green Algae	*Ap*CDS1	*Auxenochlorella protothecoides*	XP_011399129
	*Ap*CDS2	*Auxenochlorella protothecoides*	XP_011400015
	*Cr*CDS1	*Chlamydomonas reinhardtii*	EDP04329
	*Cr*CDS2	*Chlamydomonas reinhardtii*	EDP05553
	*Ot*CDS1	*Ostreococcus tauri*	CEF98602
	*Ot*CDS2	*Ostreococcus tauri*	CEF97647
Plants	*At*CDS1	*Arabidopsis thaliana*	O04928
	*At*CDS2	*Arabidopsis thaliana*	O49639
	*At*CDS3	*Arabidopsis thaliana*	Q1PE48
	*At*CDS4	*Arabidopsis thaliana*	Q94A03
	*At*CDS5	*Arabidopsis thaliana*	Q9M001
Fungi	*Cg*CDS	*Candida glabrata*	KTB16840
	*Nc*CDS	*Neurospora crassa*	EAA29473
	*Sc*CDS	*Saccharomyces cerevisiae*	P38221
	*Sp*CDS	*Schizosaccharomyces pombe*	Q9P381
Animals	*Dm*CDS	*Drosophila melanogaster*	P56079
	*Hs*CDS1	*Homo sapiens*	NP_001254
	*Hs*CDS2	*Homo sapiens*	NP_003809
Phosphatidylglycerophosphate Synthase (PGPS)			
Apicomplexans	*Ef*PGPS	*Eimeria falciformis*	EfaB_MINUS_17907.g1626
	*Nc*PGPS	*Neospora caninum*	NCLIV_063320
	*Pb*PGPS	*Plasmodium berghei*	PBANKA_0710600
	*Pf*PGPS	*Plasmodium falciparum*	PF3D7_0820200
	*Tg*PGPS	*Toxoplasma gondii*	TGGT1_246530
Kinetoplastids	*Lm*PGPS	*Leishmania major*	LmjF.07.0200
	*Tb*PGPS	*Trypanosoma brucei*	Tb927.8.1720
	*Tc*PGPS	*Trypanosoma cruzi*	TcCLB.505071.100
Bacteria	*Ec*PGPS	*Escherichia coli*	AAA98754
	*Hp*PGPS	*Helicobacter pylori*	EIE30130
Cyanobacteria	*Mv*PGPS	*Microcoleus vaginatus*	WP_006632349
	*Ss*PGPS	*Synechocystis sp. PCC6803*	ALJ68109
	*Te*PGPS	*Trichodesmium erythraeum*	ABG49777
Red Algae	*Cm*PGPS	*Cyanidioschyzon merolae*	BAM80263
Green Algae	*Cr*PGPS	*Chlamydomonas reinhardtii*	EDO97733
Plants	*At*PGPS1	*Arabidopsis thaliana*	O80952
	*At*PGPS2	*Arabidopsis thaliana*	Q9M2W3
	*Os*PGPS	*Oryza sativa*	XP_015630365
Fungi	*Cg*PGPS	*Candida glabrata*	CAG58076
	*Sc*PGPS	*Saccharomyces cerevisiae*	CAC42966
	*Sp*PGPS	*Schizosaccharomyces pombe*	Q9HDW1
Animals	*Dm*PGPS	*Drosophila melanogaster*	NP_650751
	*Dr*PGPS	*Danio rerio*	XP_003199680
	*Hs*PGPS	*Homo sapiens*	AAI08733
Cardiolipin Synthase (CLS)			
Apicomplexans	*Cp*CLS	*Cryptosporidium parvum*	cgd3_2940
	*Ef*CLS	*Eimeria falciformis*	EfaB_PLUS_56725.g2797
	*Nc*CLS	*Neospora caninum*	NCLIV_054060
	*Pb*CLS	*Plasmodium berghei*	PBANKA_0108000
	*Pf*CLS	*Plasmodium falciparum*	PF3D7_0609400
	*Tg*CLS	*Toxoplasma gondii*	TGGT1_309940
Kinetoplastids	*Lm*CLS	*Leishmania major*	LmjF.34.2110

	*Tb*CLS	*Trypanosoma brucei*	Tb927.4.2560
	*Tc*CLS	*Trypanosoma cruzi*	TcCLB.506559.40
Bacteria	*Ec*CLS1	*Escherichia coli*	ALZ68966
	*Ec*CLS2	*Escherichia coli*	ALZ69100
	*Ec*CLS3	*Escherichia coli*	WP_044697990
	*Hp*CLS	*Helicobacter pylori*	AFI01980
Cyanobacteria	*Mv*CLS	*Microcoleus vaginatus*	WP_006631652
	*Ss*CLS	*Synechocystis sp. PCC6803*	AGF52708
	*Te*CLS	*Trichodesmium erythraeum*	WP_011611499
Red Algae	*Cc*CLS	*Chondrus crispus*	XP_005712170
	*Cm*CLS	*Cyanidioschyzon merolae*	BAM81296
Green Algae	*Cr*CLS	*Chlamydomonas reinhardtii*	XP_001699073
	*Ot*CLS	*Ostreococcus tauri*	XP_003082955
Plants	*At*CLS	*Arabidopsis thaliana*	Q93YW7
	*Os*CLS	*Oryza sativa*	Q5N9A1
Fungi	*Cg*CLS	*Candida glabrata*	KTB14470
	*Sc*CLS	*Saccharomyces cerevisiae*	NP_010139
	*Sp*CLS	*Schizosaccharomyces pombe*	CAB16578
Animals	*Dm*CLS	*Drosophila melanogaster*	NP_651418
	*Dr*CLS	*Danio rerio*	NP_998096
	*Hs*CLS	*Homo sapiens*	ABD46888
Phosphatidylserine Decarboxylase (PSD)			
Apicomplexans	*Cp*PSD	*Cryptosporidium parvum*	cgd3_2100
	*Ef*PSD1	*Eimeria falciformis*	EfaB_MINUS_22450.g1948
	*Ef*PSD2	*Eimeria falciformis*	EfaB_MINUS_1072.g165
	*Nc*PSD1	*Neospora caninum*	NCLIV_047160
	*Nc*PSD2	*Neospora caninum*	NCLIV_036570
	*Pb*PSD	*Plasmodium berghei*	PBANKA_0828700
	*Pf*PSD	*Plasmodium falciparum*	PF3D7_0927900
	*Tg*PSD1	*Toxoplasma gondii*	TGGT1_225550
	*Tg*PSD2	*Toxoplasma gondii*	TGGT1_269920
Kinetoplastids	*Lm*PSD	*Leishmania major*	LmjF.35.4590
	*Tb*PSD	*Trypanosoma brucei*	Tb927.9.10080
Bacteria	*Ec*PSD	*Escherichia coli*	AAA83896
	*Se*PSD	*Salmonella enterica*	CBY98536
	*Yk*PSD	*Yersinia kristensenii*	AJJ36856
Cyanobacteria	*Ma*PSD	*Microcystis aeruginosa*	WP_002731706
	*Pm*PSD	*Prochlorococcus marinus*	ABM78815
	*Xs*PSD	*Xenococcus sp. PCC 7305*	ELS02409
Red Algae	*Cc*PSD	*Chondrus crispus*	CDF41167
	*Cm*PSD	*Cyanidioschyzon merolae*	BAM80594
Green Algae	*Ap*PSD	*Auxenochlorella protothecoides*	KFM28115
	*Ts*PSD	*Tetraselmis sp. GSL018*	JAC78653
Plants	*At*PSD1	*Arabidopsis thaliana*	Q84V22
	*At*PSD2	*Arabidopsis thaliana*	F4KAK5
	*Os*PSD1	*Oryza sativa*	Q10T43
	*Os*PSD2	*Oryza sativa*	Q5JN42
Fungi	*Cg*PSD1	*Candida glabrata*	KTB16854
	*Cg*PSD2	*Candida glabrata*	KTB25137
	*Sc*PSD1	*Saccharomyces cerevisiae*	P39006
	*Sc*PSD2	*Saccharomyces cerevisiae*	P53037
Animals	*Dm*PSD	*Drosophila melanogaster*	AAF56228
	*Dr*PSD	*Danio rerio*	NP_001073170
	*Hs*PSD	*Homo sapiens*	NP_001313340
Phosphatidylserine Synthase (PSS) / Phosphatidylthreonine Synthase (PTS)			
Apicomplexans	*Cp*PSS	*Cryptosporidium parvum*	cgd1_1110
	*Ef*PSS	*Eimeria falciformis*	EfaB_PLUS_1974.g212

	*Ef*PTS	*Eimeria falciformis*	EfaB_MINUS_800.g81
	*Nc*PSS	*Neospora caninum*	NCLIV_026010
	*Nc*PTS	*Neospora caninum*	NCLIV_034110
	*Pb*PSS	*Plasmodium berghei*	PBANKA_1142700
	*Pf*PSS	*Plasmodium falciparum*	PF3D7_1366800
	*Tg*PSS	*Toxoplasma gondii*	TGGT1_261480
	*Tg*PTS	*Toxoplasma gondii*	TGGT1_273540
Kinetoplastids	*Lm*PSS	*Leishmania major*	LmjF.14.1200
	*Tb*PSS	*Trypanosoma brucei*	Tb927.7.3760
	*Tc*PSS	*Trypanosoma cruzi*	TcCLB.509937.30
Bacteria	*Bs*PSS	*Bacillus subtilis*	BAA07225
	*Hp*PSS	*Helicobacter pylori*	AAC45587
	*Mt*PSS	*Mycobacterium tuberculosis*	GAA44221
Green Algae	*Ap*PSS	*Auxenochlorella protothecoides*	KFM28612
	*Cv*PSS	*Chlorella variabilis*	EFN55835
Plants	*At*PSS	*Arabidopsis thaliana*	AEE29266
	*Os*PSS	*Oryza sativa*	AAT58731
	*Zm*PSS	*Zea mays*	ACG35885
Fungi	*Cg*PSS	*Candida glabrata*	KTB22122
	*Sc*PSS	*Saccharomyces cerevisiae*	BAA00121
	*Sp*PSS	*Schizosaccharomyces pombe*	O94584
Animals	*Dm*PSS	*Drosophila melanogaster*	AAF51622
	*Hs*PSS1	*Homo sapiens*	AAH04390
	*Hs*PSS2	*Homo sapiens*	AAH01210
Phosphatidic Acid Phosphatase (PAP)			
Apicomplexans	*Cp*PAP	*Cryptosporidium parvum*	cgd8_5200
	*Ef*PAP	*Eimeria falciformis*	EfaB_MINUS_22960.g1971
	*Nc*PAP	*Neospora caninum*	NCLIV_063870
	*Pf*PAP	*Plasmodium falciparum*	PF3D7_0805600
	*Tg*PAP	*Toxoplasma gondii*	TGGT1_247360
Kinetoplastids	*Lm*PAP	*Leishmania major*	LmjF.18.0440
	*Tb*PAP1	*Trypanosoma brucei*	Tb927.8.480
	*Tb*PAP2	*Trypanosoma brucei*	Tb927.10.13930
	*Tb*PAP3	*Trypanosoma brucei*	Tb927.10.13400
	*Tc*PAP1	*Trypanosoma cruzi*	TcCLB.503809.110
	*Tc*PAP2	*Trypanosoma cruzi*	TcCLB.511277.370
	*Tc*PAP3	*Trypanosoma cruzi*	TcCLB.511277.359
Bacteria	*Hp*PAP	*Helicobacter pylori*	EJB76973
	*Se*PAP	*Salmonella enterica*	AFH43770
Cyanobacteria	*Ce*PAP	*Crinalium epipsammum*	WP_051035457
	*Mv*PAP	*Microcoleus vaginatus*	EGK88082
Red Algae	*Cm*PAP1	*Cyanidioschyzon merolae*	BAM82646
	*Cm*PAP2	*Cyanidioschyzon merolae*	BAM82332
	*Gs*PAP1	*Galdieria sulphuraria*	EME32237
	*Gs*PAP2	*Galdieria sulphuraria*	EME28653
Green Algae	*Ot*PAP	*Ostreococcus tauri*	CAL52864
	*Vc*PAP	*Volvox carteri*	EFJ45722
Plants	*At*PAP1	*Arabidopsis thaliana*	AEE73835
	*At*PAP2	*Arabidopsis thaliana*	BAB47575
	*At*PAP3	*Arabidopsis thaliana*	AEE76067
	*At*PAP4	*Arabidopsis thaliana*	BAB47574
	*At*PAP5	*Arabidopsis thaliana*	AEE78725
	*At*PAP6	*Arabidopsis thaliana*	AED 90554
	*At*PAP7	*Arabidopsis thaliana*	AEE84623
Fungi	*Cg*PAP1	*Candida glabrata*	KTB24721
	*Cg*PAP2	*Candida glabrata*	KTB19937
	*Sc*PAP1	*Saccharomyces cerevisiae*	AAS56070

	*Sc*PAP2	*Saccharomyces cerevisiae*	AAT93210
Animals	*Hs*PAP1	*Homo sapiens*	NP_003702
	*Hs*PAP2	*Homo sapiens*	NP_003704
	*Hs*PAP3	*Homo sapiens*	NP_003703
	*Hs*PAP4	*Homo sapiens*	NP_001025230
	*Hs*PAP5	*Homo sapiens*	AAI06015
	*Hs*PAP6	*Homo sapiens*	AAH38108
	*Hs*PAP7	*Homo sapiens*	AAH06362
	*Hs*PAP8	*Homo sapiens*	Q8TBJ4
	*Hs*PAP9	*Homo sapiens*	Q96GM1
	*Hs*PAP10	*Homo sapiens*	Q6T4P5
	*Hs*PAP11	*Homo sapiens*	Q7Z2D5
	*Hs*PAP12	*Homo sapiens*	Q32ZL2
Diacylglycerol Kinase (DGK)			
Apicomplexans	*Cp*DGK1	*Cryptosporidium parvum*	cgd4_4340
	*Cp*DGK2	*Cryptosporidium parvum*	cgd8_2390
	*Cp*DGK3	*Cryptosporidium parvum*	cgd3_2630
	*Ef*DGK1	*Eimeria falciformis*	EfaB_MINUS_7048.g670
	*Ef*DGK2	*Eimeria falciformis*	EfaB_MINUS_36188.g2575
	*Ef*DGK3	*Eimeria falciformis*	EfaB_MINUS_11882.g1052
	*Nc*DGK1	*Neospora caninum*	NCLIV_022470
	*Nc*DGK2	*Neospora caninum*	NCLIV_027060
	*Nc*DGK3	*Neospora caninum*	NCLIV_015910
	*Pb*DGK1	*Plasmodium berghei*	PBANKA_1334600
	*Pb*DGK2	*Plasmodium berghei*	PBANKA_0831200
	*Pf*DGK1	*Plasmodium falciparum*	PF3D7_1471400
	*Pf*DGK2	*Plasmodium falciparum*	PF3D7_0930500
	*Tg*DGK1	*Toxoplasma gondii*	TGGT1_202460
	*Tg*DGK2	*Toxoplasma gondii*	TGGT1_259830
	*Tg*DGK3	*Toxoplasma gondii*	TGGT1_239250
Kinetoplastids	*Lm*DGK1	*Leishmania major*	LmjF.16.1290
	*Lm*DGK2	*Leishmania major*	LmjF.35.5370
	*Tb*DGK1	*Trypanosoma brucei*	Tb927.8.5140
	*Tc*DGK1	*Trypanosoma cruzi*	TcCLB.506575.60
	*Tc*DGK2	*Trypanosoma cruzi*	TcCLB.510329.290
Bacteria	*Bs*DGK	*Bacillus subtilis*	AAA70044
	*Ec*DGK	*Escherichia coli*	CUU96332
	*Hp*DGK1	*Helicobacter pylori*	EIE31169
	*Hp*DGK2	*Helicobacter pylori*	KRV53075
Cyanobacteria	*Mv*DGK	*Microcoleus vaginatus*	WP_039887027
	*Ss*DGK	*Synechocystis sp. PCC6803*	ALJ68494
	*Te*DGK	*Trichodesmium erythraeum*	WP_011614234
Red Algae	*Cm*DGK	*Cyanidioschyzon merolae*	BAM80462
	*Gs*DGK1	*Galdieria sulphuraria*	EME26535
	*Gs*DGK2	*Galdieria sulphuraria*	EME28250
Green Algae	*Cr*DGK	*Chlamydomonas reinhardtii*	EDP07029
	*Ot*DGK1	*Ostreococcus tauri*	CEF97562
	*Ot*DGK2	*Ostreococcus tauri*	CEF98712
Plants	*At*DGK1	*Arabidopsis thaliana*	CAB62604
	*At*DGK2	*Arabidopsis thaliana*	AAR28755
	*At*DGK3	*Arabidopsis thaliana*	AEC06799
	*At*DGK4	*Arabidopsis thaliana*	AED 96936
	*At*DGK5	*Arabidopsis thaliana*	AEC07096
	*At*DGK6	*Arabidopsis thaliana*	AEE85443
	*At*DGK7	*Arabidopsis thaliana*	AEE85754
Fungi	*Cg*DGK	*Candida glabrata*	KTB25101
	*Sc*DGK	*Saccharomyces cerevisiae*	DAA11076

	*Sp*DGK	*Schizosaccharomyces pombe*	P87170
Animals	*Hs*DGK1	*Homo sapiens*	P23743
	*Hs*DGK2	*Homo sapiens*	Q9Y6T7
	*Hs*DGK3	*Homo sapiens*	P49619
	*Hs*DGK4	*Homo sapiens*	Q16760
	*Hs*DGK5	*Homo sapiens*	P52429
	*Hs*DGK6	*Homo sapiens*	Q13574
	*Hs*DGK7	*Homo sapiens*	Q86XP1
	*Hs*DGK8	*Homo sapiens*	P52824
	*Hs*DGK9	*Homo sapiens*	O75912
	*Hs*DGK10	*Homo sapiens*	Q5KSL6
Choline/Ethanolamine Phosphotransferase (CEPT)			
Apicomplexans	*Cp*CEPT1	*Cryptosporidium parvum*	cgd4_2790
	*Cp*CEPT2	*Cryptosporidium parvum*	cgd4_390
	*Ef*CEPT1	*Eimeria falciformis*	EfaB_MINUS_56725.g2919
	*Ef*CEPT2	*Eimeria falciformis*	EfaB_MINUS_15745.g1427
	*Ef*CEPT3	*Eimeria falciformis*	EfaB_MINUS_25458.g2174
	*Nc*CEPT1	*Neospora caninum*	NCLIV_029590
	*Nc*CEPT2	*Neospora caninum*	NCLIV_025720
	*Nc*CEPT3	*Neospora caninum*	NCLIV_006960
	*Pb*CEPT	*Plasmodium berghei*	PBANKA_1127000
	*Pf*CEPT	*Plasmodium falciparum*	PF3D7_0628300
	*Tg*CEPT1	*Toxoplasma gondii*	TGGT1_257510
	*Tg*CEPT2	*Toxoplasma gondii*	TGGT1_261760
	*Tg*CEPT3	*Toxoplasma gondii*	TGGT1_276190
Kinetoplastids	*Lm*CEPT	*Leishmania major*	LmjF.36.5900
	*Tb*CEPT	*Trypanosoma brucei*	Tb927.10.8900
	*Tc*CEPT	*Trypanosoma cruzi*	TcCLB.509791.150
Red Algae	*Gs*CEPT1	*Galdieria sulphuraria*	EME27119
	*Gs*CEPT2	*Galdieria sulphuraria*	EME32110
Green Algae	*Ot*CEPT1	*Ostreococcus tauri*	XP_003080347
	*Ot*CEPT2	*Ostreococcus tauri*	CEF97354
Plants	*At*CEPT1	*Arabidopsis thaliana*	O82567
	*At*CEPT2	*Arabidopsis thaliana*	O82568
	*Zm*CEPT1	*Zea mays*	XP_008649197
	*Zm*CEPT2	*Zea mays*	AFW69854
Fungi	*Cg*CEPT1	*Candida glabrata*	KTB18544
	*Cg*CEPT2	*Candida glabrata*	KTB25778
	*Sc*CEPT1	*Saccharomyces cerevisiae*	P22140
	*Sc*CEPT2	*Saccharomyces cerevisiae*	AAA63571
Animals	*Dr*CEPT	*Danio rerio*	NP_001103187
	*Hs*CEPT1	*Homo sapiens*	NP_006081
	*Hs*CEPT2	*Homo sapiens*	AAH50429
Phosphatidylinositol Synthase (PIS)			
Apicomplexans	*Ef*PIS	*Eimeria falciformis*	EfaB_PLUS_6035.g572
	*Nc*PIS	*Neospora caninum*	NCLIV_002660
	*Pb*PIS	*Plasmodium berghei*	PBANKA_1414100
	*Pf*PIS	*Plasmodium falciparum*	PF3D7_1315600
	*Tg*PIS	*Toxoplasma gondii*	TGGT1_207710
Kinetoplastids	*Lm*PIS	*Leishmania major*	LmjF.26.2480
	*Tb*PIS	*Trypanosoma brucei*	Tb927.9.1610
	*Tc*PIS	*Trypanosoma cruzi*	TcCLB.503925.80
Red Algae	*Cc*PIS	*Chondrus crispus*	CDF39583
	*Cm*PIS	*Cyanidioschyzon merolae*	BAM80990
	*Gs*PIS	*Galdieria sulphuraria*	XP_005703945
Green Algae	*Ap*PIS	*Auxenochlorella protothecoides*	KFM23061
	*Cr*PIS	*Chlamydomonas reinhardtii*	EDP06395

	*Ot*PIS	*Ostreococcus tauri*	CAL56685
Plants	*At*PIS	*Arabidopsis thaliana*	Q8LBA6
	*Os*PIS	*Oryza sativa*	CAC37011
	*Zm*PIS	*Zea mays*	NP_001105559
Fungi	*Cg*PIS	*Candida glabrata*	KTB24417
	*Sc*PIS	*Saccharomyces cerevisiae*	AAA34876
	*Sp*PIS	*Schizosaccharomyces pombe*	Q10153
Animals	*Dm*PIS	*Drosophila melanogaster*	AAF48491
	*Dr*PIS	*Danio rerio*	AAT68039
	*Hs*PIS	*Homo sapiens*	AAB94860

Appendix 6. *Tg*CDS2 harbors bipartite signal and transit peptides.

The positions of nucleotides and amino acids of *Tg*CDS2 N-terminal extension are indicated along both margins. All methionine residues are marked in bold. The bipartite peptide sequence starts from the second methionine located in the extended N-terminus. The putative signal and transit peptides are depicted in green and yellow colors, respectively.

```
   1 ATGGAGGAAACGCAGACTTCAGAGAAGCCGACAACTGCCGAGGTCAAGAGATCGACCGCGGCAGGAAGTCCCTCATTTCAGAAAACCAAG  90
   1  M  E  E  T  Q  T  S  E  K  P  T  T  A  E  V  K  R  S  T  A  A  G  S  P  S  F  Q  K  T  K  30
  91 TTTGAAATTGTGACACACAGAAAACGACCCTTGCAAGTTGACTCCACTTTTACAAAAACAGTGGAAGCCTCCGCGAGAGGTCGAGTGCTC 180
  31  F  E  I  V  T  H  R  K  R  P  L  Q  V  D  S  T  F  T  K  T  V  E  A  S  A  R  G  R  V  L  60
 181 TCTTCCCTCTCTTCAACGGTCTCCGAGGCTTTGTCTCCTAACTGTCTCTTTCAAGCGATTCCTTCCCCCCATGGGTCTTCTCGTCTTCCT 270
  61  S  S  L  S  S  T  V  S  E  A  L  S  P  N  C  L  F  Q  A  I  P  S  P  H  G  S  S  R  L  P  90
 271 CGACTCCGGCCTCTGCATCCTTCTTCCGGCAAGTCTTCTCCAACTGCTTCTCTCTCTGCCTCGTCTGCTGCTCGCGCCTGCTCTTCTTCC 360
  91  R  L  R  P  L  H  P  S  S  G  K  S  S  P  T  A  S  L  S  A  S  S  A  A  R  A  C  S  S  S 120
 361 TCGACTCCCTCTTCCAGGTGGATGCGCGTCCATTCGTCGTATGCATTTTCATCGCTTCCTTGGCTGTTCGTTTCCGCGTCATTCCTTCTG 450
 121  S  T  P  S  S  R  W  M  R  V  H  S  S  Y  A  F  S  S  L  P  W  L  F  V  S  A  S  F  L  L 150
 451 ATTCACTCGACTCGCAATGGCGTCCTTCTCAGCGACGGCGTTCGCGCGGTGTCTCCGTCGTCTTCGTCCTTCCCTCATCACAGAATTTCC 540
 151  I  H  S  T  R  N  G  V  L  L  S  D  G  V  R  A  V  S  P  S  S  S  S  F  P  H  H  R  I  S 180
 541 TTCTCTTCGTCGTCTTCTCTCGCTTCTTCTTCTCTTGCGTTTCTTTCTCTAAGGGACCTCTCTGCCGCCAGGATGGATTCGTCGTCTGCA 630
 181  F  S  S  S  S  S  L  A  S  S  S  L  A  F  L  S  L  R  D  L  S  A  A  R  M  D  S  S  S  A 210
 631 GATCCTCTCGTCTCTCAGCAGACCCTGCTGTCTCGGTCTCCACGTCCTTCTCGTTCTTCTTCTCCCTCTCCCTCTTTGTGTTCTCTCTCT 720
 211  D  P  L  V  S  Q  Q  T  L  L  S  R  S  P  R  P  S  R  S  S  S  P  S  P  S  L  C  S  L  S 240
 721 TCCTCTTCTACGGCTTCTCCCTCCTCTCCTTCTTCCTCGTTCTCCTCCCCTCTGTCTTTGTTTTCTTCTCCTCTCTCTTCTCGTTCGGCA 810
 241  S  S  S  T  A  S  P  S  S  P  S  S  S  F  S  S  P  L  S  L  F  S  S  P  L  S  S  R  S  A 270
 811 GATCGTTCGGCCTCTTCTTCTCGGCTCTCGGCCGCCTCACTTCCGCGGCTCCCCCCGTCTTTTGTCGCCGTCTCACCGGGACCTCCTGTC 900
 271  D  R  S  A  S  S  S  R  L  S  A  A  S  L  P  R  L  P  P  S  F  V  A  V  S  P  G  P  P  V 300
 901 CGTCTCTGTCCTTCGTCGCAGCTTTCACTCTCTTCTCTGCACTCGCCTGTATCAGGAACAAGCGCGCCGAAGAGCAATCGGCAGCACCCT 990
 301  R  L  C  P  S  S  Q  L  S  L  S  S  L  H  S  P  V  S  G  T  S  A  P  K  S  N  R  Q  H  P 330
 991 GCCTCGGTATGTCCTCCCCTTCTTTCTCCTCTCTCGTCAGAACTGTACTCTTCTGTGCATGCAGGCAGGAACGCAGCGGGTGCTGGCTCT 1080
 331  A  S  V  C  P  P  L  L  S  P  L  S  S  E  L  Y  S  S  V  H  A  G  R  N  A  A  G  A  G  S 360
1081 CAGAGAGGACGAATGCCGAAGAAAAAATCAGGAAAAACACGCATGCAGCCTTCGCCTTCCTCTCCTTCTTCTTCTCCTTCTCCTTCTTCT 1170
 361  Q  R  G  R  M  P  K  K  K  S  G  K  T  R  M  Q  P  S  P  S  S  P  S  S  S  P  S  P  S  S 390
1171 CCTTCTTCTTCTTCTTCATCTCCTTCTGGTTCTTCTCTTGGTTCGCCGTCTTCGTCTCCTGCTTCGTCTCGTTCGGAAGCGTTTGGTGAT 1260
 391  P  S  S  S  S  S  S  P  S  G  S  S  L  G  S  P  S  S  S  P  A  S  S  R  S  E  A  F  G  D 420
1261 TCAGCTTCGGTGGAGGCATGTCACTGGGGACAGCAAGAGAGGGAAGAAAGAAAACGTTCGGTTTCGACAGGGCGAGGCGCGTCCTTTCTT 1350
 421  S  A  S  V  E  A  C  H  W  G  Q  Q  E  R  E  E  R  K  R  S  V  S  T  G  R  G  A  S  F  L 450
1351 GCATGTCTGCGAAAAGCGAAAGCGCAGATCGCTTCTCGCATGCACCTCAGTGCCTCTCCACGCCTTTGGAATTCGATATCTCGAGTTCTT 1440
 451  A  C  L  R  K  A  K  A  Q  I  A  S  R  M  H  L  S  A  S  P  R  L  W  N  S  I  S  R  V  L 480
1441 TCTCTTGCTCCTCTCCCCTCATGCTCCAGTGCGTCTGCCTCGATGTCGCTCGCTCCTTCTCCTGCCGCCCCGTCTTCTTCTTCTTCTTCC 1530
 481  S  L  A  P  L  P  S  C  S  S  A  S  A  S  M  S  L  A  P  S  P  A  A  P  S  S  S  S  S  S 510
1531 TCTCCGCCGTCGTCTTCTTCTTCTCTTTCCTCTCGCTTTTCTTCCTTG                                        1578
 511  S  P  P  S  S  S  S  S  L  S  S  R  F  S  S  L                                        526
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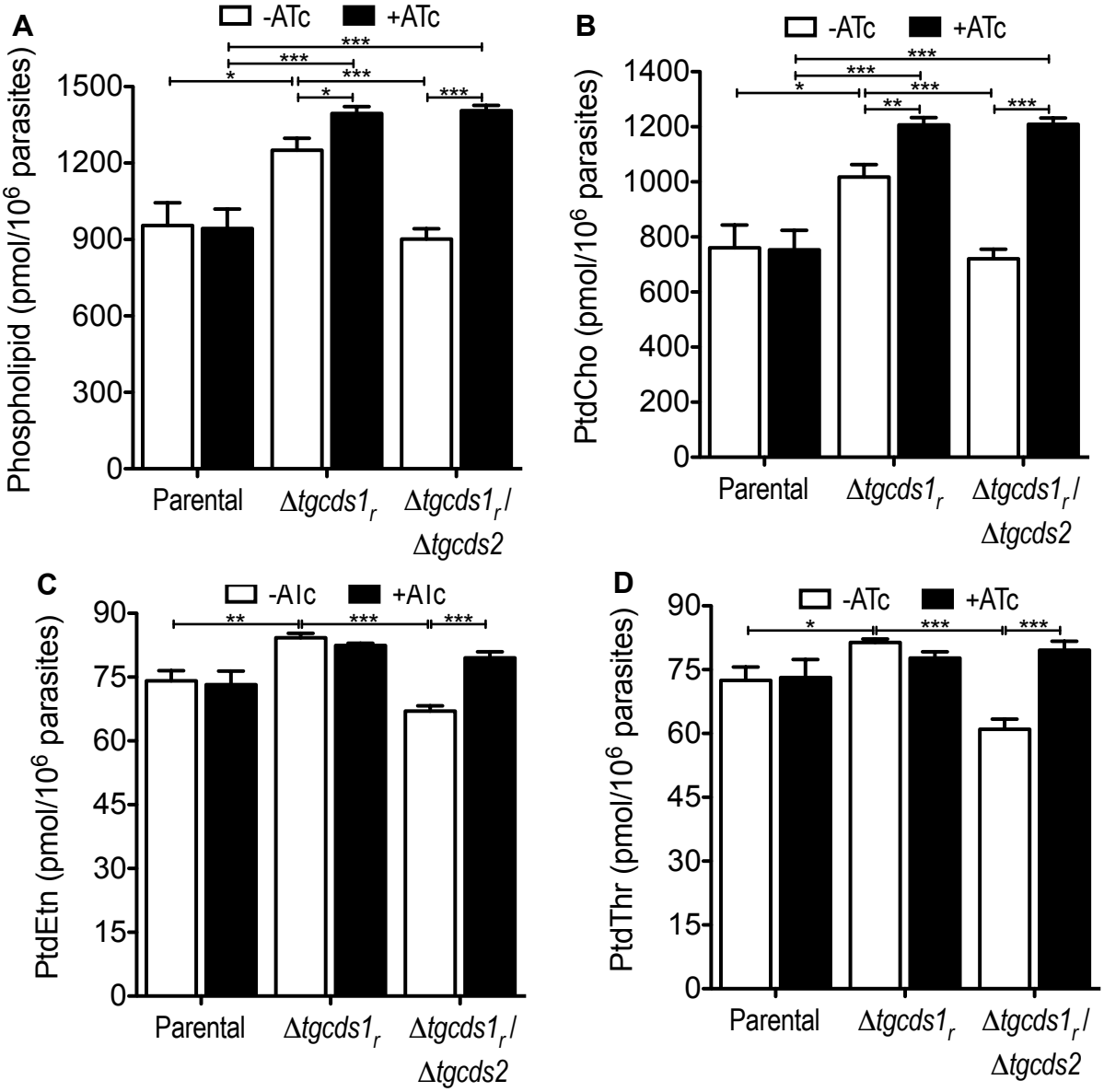
Appendix 7. Phospholipid profiles of the Δ*tgcds1*$_r$ and Δ*tgcds1*$_r$/Δ*tgcds2* mutants.

Lipids isolated from tachyzoites were analyzed by HPLC/MS. Major phospholipids identified were PtdCho, PtdEtn, PtdThr, PtdIns, PtdSer and PtdGro. (**A**) Cumulative sum of all lipids in the indicated strains cultured with or without ATc. Other panels illustrate PtdCho (**B**), PtdEtn (**C**) and PtdThr (**D**). PtdIns, PtdSer and PtdGro are depicted in Figure 14. Values are means with SEM from six independent assays (*p < 0.05, **p < 0.01, ***p < 0.001).



Appendix 8. Vector sequences.

(**A**) *pGRA1-UPKO-EfPTS*

Function: Ectopic expression of selected *E. falciformis* enzymes in *T. gondii* tachyzoites.

*Ef*PTS-HA could be replaced with other *E. falciformis* enzymes of interest.

*Nsi*I and *Pac*I sites for inserting ORF-HA of selected enzymes

```
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(**B**) *pG152-TgCDS1*
Function: 3' HA tagging of *Tg*CDS1.

*Nae*I site for linearizing the vector before transfection

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CTTCGCGAATGCGTCGAGATCGTACCCTGTACTTCCAATCCAATTTAAT

(**C**) *pG152-TgCDS2*
Function: 3' HA tagging of *Tg*CDS2.

*Tg*CDS2-3COS | HA | *Tg*SAG1-3UTR | p*TgDHFR-TS* | *Tg*HXGPRT-ORF | *Tg*DHFR-TS-3UTR

*Xho*I site for linearizing the vector before transfection

GCTCTCCCGGTGTCTGTCGTCTTTCTGCTAGCTTCGCTGCTTCTCTCTTCACCCTCGACGAAGACCATCGCAGACATTTCAGCATCCATCTTCT
CCCTGATTTGGTGTGTCGTCCTTCCTTCCTTCCTTCTGGGTAAAACTGCGTTTTCTCCGCATCTTGCCTGACGATGCTTTCTCCTCGCCTCTCGGCCG
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AGATCGTACCCTGTACTTCCAATCCAATTTAAT

(**D**) *pG152-TgPGPS*
Function: 3' HA tagging of *Tg*PGPS.

*Sac*I site for linearizing the vector before transfection

GCAGAAGGCGGAGAAGAGCGTTTCGCGGGTCTTGATTCGGAGGCGAAGAACGGGGGAAAGGCCGCGAGGTCAAGATCTAGAGGAGAAGACGAAG
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ACTTCGCGAATGCGTCGAGATCGTACCCTGTACTTCCAATCCAATTTAAT

(**E**) *pTKO-CAT-TgCDS1*$^{398\text{-}1068}$*-Myc*

Function: Ectopic expression of *Tg*CDS1 without N-terminal extension and with C-terminal Myc tag.

NsiI and *PacI* sites for inserting *Tg*CDS1$_{398\text{-}1068}$-Myc
NotI site for linearizing the vector before transfection

CCACCGGTCACCTGGCGTCTCAGCTGCGCGAGGCACCCCTCTCATAGCGTGGTACTCGTCACGAATACCAATCGCTGGGGTCGCGGCGGGAGGA
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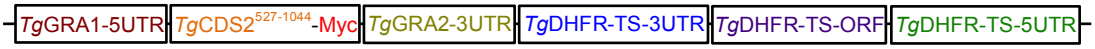
A

(**F**) *pTKO-DHFR-TS-TgCDS2*$^{527\text{-}1044}$*-Myc*

Function: Ectopic expression of *Tg*CDS2 without N-terminal extension and with C-terminal Myc tag.



NsiI/*SbfI* and *PacI* sites for inserting *Tg*CDS2$_{527\text{-}1044}$-Myc
NotI site for linearizing the vector before transfection

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(**G**) *pTETO7SAG1-UPKO-Myc-TgCDS1-HA* (partial)
Function: Ectopic expression of *Tg*CDS1 with N-terminal Myc tag and C-terminal HA tag.

*Nco*I/*BspH*I and *Pac*I sites for inserting Myc-*Tg*CDS1-HA
*Not*I site for linearizing the vector before transfection

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(**H**) *pTETO7SAG1-UPKO-Myc-TgCDS2-HA* (partial)
Function: Ectopic expression of *Tg*CDS2 with N-terminal Myc tag and C-terminal HA tag.

*Nco*I/*Bsp*HI and *Pac*I sites for inserting Myc-*Tg*CDS2-HA
*Not*I site for linearizing the vector before transfection

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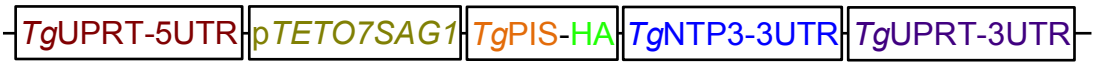
(**I**) *pTETO7SAG1-UPKO-TgPIS-HA* (partial)
Function: Ectopic expression of *Tg*PIS with C-terminal HA tag.



*Nco*I and *Pac*I sites for inserting *Tg*PIS-HA
*Not*I site for linearizing the vector before transfection

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(**J**) *pTETO7SAG1-UPKO-TgCDS1-HA* (partial)
Function: Ectopic expression of *Tg*CDS1-HA under the control of p*TETO7SAG1* promoter.

*Nco*I/*Bsp*HI and *Pac*I sites for inserting *Tg*CDS1-HA
*Not*I site for linearizing the vector before transfection

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CCTCGGCAAAAGTGTAAGCAGGCGGCCGC

(**K**) *pTKO-DHFR-TS-TgCDS1-KO*

Function: Deletion of *TgCDS1* locus by double homologous recombination.

*Xcm*I and *Spe*I sites for inserting *Tg*CDS1-5UTR
*Hind*III and *Apa*I sites for inserting *Tg*CDS1-3UTR
*Apa*I site for linearizing the vector before transfection

CCACCGGTCACCTGGCGAATCGTGTTTGTTTCCTCTTTACAGTTACCGTGGCAGTTGACAGGCAACTCTCGGAACTATTAAGTCGCTATGCAGT
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GA

(**L**) *pTUB8-CAT-TgCDS2-KO*

Function: Deletion of *TgCDS2* locus by double homologous recombination.

*Apa*I sites for inserting *Tg*CDS2-5UTR
*Xho*I and *Xba*I sites for inserting *Tg*CDS2-3UTR
*Xba*I site for linearizing the vector before transfection

GGGCCCAGTCCTGTCGATTTTCTCCCTGCATGCGCGCAGTGGACAGAGCTGAAGAAAAACGCCAGACCGACCGCCGGTCCAGCAGCAGCTTGGT
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List of conferences and courses

Presentations in conferences

04.2016, IRI Symposium: Molecular Interactions in Malaria, Berlin/Germany;

03.2016, 27th Annual DGP Meeting, Göttingen/Germany;

09.2014, 11th International Coccidiosis Conference, Dresden/Germany;

09.2014, 12th Euro Fed Lipid Congress, Montpellier/France;

05.2014, 3rd European Symposium on Microbial Lipids: Model Organisms and Biodiversity, Hamburg/Germany;

01.2014, ZIBI Graduate School Scientific Thinking, Berlin/Germany;

06.2013, 12th International Congress on Toxoplasmosis, Oxford/UK;

03.2013, IFoTox-2013 Interdisciplinary Form on Toxoplasmosis, Göttingen/Germany;

09.2012, 23rd Annual Molecular Parasitology Meeting, Woods Hole/USA;

03.2012, 23rd Annual DGP Meeting, Heidelberg/Germany;

2011-2015, ZIBI Graduate School Retreats, Berlin/Germany.

Practical and soft skill courses

06.2013, Communication at Conferences;

03.2012, 6th Short Course for Young Parasitologists;

03.2012, FACS Calibur;

09.2011, Publishing Research Articles;

06.2011, Work-Life-Balance in the Academic World;

06.2011, Grant Application Writing;

05.2011, Analysis of Innate Immune Recognition in Bacterial Infections;

01.2011, Science-Ethics-Politics: Agenda Setting in Science;

09.2010, Presenting Professionally.

List of publications

During PhD period:

Kong P, Ufermann CM, Zimmermann DL, Yin Q, Suo X, Helms BJ, Brouwers JF and Gupta N. Two phylogenetically and compartmentally distinct CDP-diacylglycerol synthases cooperate for lipid biogenesis in *Toxoplasma gondii*. *J Biol Chem*. 2017;292(17):7145-59.

Kong P, Lehmann MJ, Helms JB, Brouwers JF and Gupta N. Exclusive phospholipid expression and autonomous membrane biogenesis in *Eimeria* indicate a host-independent lifestyle of apicomplexan sporozoites. (in submission)

During master period:

Kong P, Wang L, Zhang H, Song X, Zhou Z, Yang J, Qiu L, Wang L and Song L. A novel C-type lectin from bay scallop *Argopecten irradians* (AiCTL-7) agglutinating fungi with mannose specificity. *Fish Shellfish Immunol*. 2011;30(3):836-44.

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Zhou Z, Yang J, Wang L, Zhang H, Gao Y, Shi X, Wang M, **Kong P**, Qiu L and Song L. A dopa decarboxylase modulating the immune response of scallop *Chlamys farreri*. *PLoS One*. 2011;6(4):e18596.

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Selbständigkeitserklärung

Hiermit erkläre ich an Eides statt, die vorliegende Dissertation selbstständig und angefertigt und keine anderen als die angegebenen Hilfsmittel verwendet zu haben.

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Ort, Datum Unterschrift